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Functional analysis of AtRPD3B, a RPD3-type histone deacetylase, in Arabidopsis

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ABSTRACT

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Histone acetylation is modulated through the action of histone acetyltransferases and deacetylases, which play key roles in the regulation of eukaryotic gene expression. The RPD3 group of histone deacetylases constitutes the first class of histone deacetylases (HDACs) in eukaryotes.

In yeast and mammalian cells, it was found that RPD3 histone deacetylases are involved in the ageing and development. In contrast much less is known about the function of RPD3s in plant. Here I studied AtRPD3B, a RPD3-type histone deacetylase in *Arabidopsis thaliana*, to define its role in plant development and signal transduction pathways. Using the *AtRPD3B:GUS* transgenic *Arabidopsis* plants, I demonstrated that the *AtRPD3B* is induced by the plant hormones, JA, ET and GA and by wounding. Sequence analysis indicated that the *AtRPD3B* promoter contains multiple hormones and stress responsive motifs. Using 5' *AtRPD3B* promoter deletion assay, the essential regulatory region was found in the -757 bp to -374 bp upstream of ATG translational start condon. Analysis of the *AtRPD3B-RNAi* plants and the *axe1-5* mutant plants suggested that AtRPD3B is involved in the flowering and senescence in *Arabidopsis*. The SAG gene, whose expression has previously been shown to be instrumental for the progression of senescence, was found to be downregulated in the *AtRPD3B* mutants. Based on the previous reports of JA involvement in senescence, the identification of *AtRPD3B* induction by JA, and the down-regulation of JA-responsive genes in the AtRPD3B mutants, we proposed that AtRPD3B might be involved in senescence via the JA pathway. AtRPD3B is involved in autonomous flowering pathway and promotes plant flowering by upregulating *FLC* and downregulating *SOC1*. This study provided evidence that AtRPD3B plays a role in the flowering and senescence in *Arabidopsis*.

ABBREVIATIONS

ABRE: abscisic acid responsive element

AG: AGAMOUS

AP2: PETALA2

AP3: APETALA3

APl: APETALA1

CAL: CAULJFLOWR

***cev1*:** constitutive overexpression of VSP1

CO: CONSTANS

COI1: coronatine insensitive1

Col: Columbia

CRY2: CRYPTOCHROME2/FHA

DAE: day after emergence

***ein2*:** ethylene insensitive 2

ELF3: EARLY FLOWERING3

EREs: ethylene responsive elements

ERF1: ethylene responsive factor

ET: ethylene

***etr1*:** ethylene responsive

FLC: flowering locus C

FRI: frigida

FT: flowering time

GA: gibberellin

GFP: green fluorescent protein

GI: GIGANTEA

GUS: the beta-glucuronidase

HAT: histone acetyl transferases

HDAC: histone deacetylase

JA: jasmonic acid

JAR1: JA resistant1

JIN1: JA insensitive1

LFY: LEAFY

LHY: LATE ELONGATED HYPOCOTL

MADS-box: MCM1-Agamous-Deficiens-SRF

MS: Murashige and Skoog

PAGs: photosynthesis associated genes

P-box: gibberellin responsive element

PDF1.2: plant defensin 1.2

PI: PISTILLATA

PR1: pathogenesis related gene

PSII efficiency: photochemical efficiency of photosystem II

RNAi: RNA interference

RPD3: reduced potassium dependancy3

RPS17: ribosomal protein small subunit 17

SA: salicylic acid

SAGs: senescence-associated genes

SCF: SKP1-Cullin-F-box

SOC1: suppressor of overexpression of CO 1

UBQ: ubiquitin

VRN2: vernalization2

VSP: vegetative storage protein

Ws: Wassilewskija

WT: wild type

WUN: wounding responsive element

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INTRODUCTION

Flowering plants undergo sequential phases in their life cycle, beginning with embryogenesis, progressing into the juvenile and the reproductive phase, respectively. Subsequently, programmed cell death is activated resulting in senescence and death of the plant. All of these physiological events can progress efficiently only if stringent control is maintained over the spatial and temporal expression of the plant genome. Regulatory control of this nature can be exercised at the level of transcription, whereby genes encoding proteins essential for specific developmental pathways are upregulated and genes encoding proteins not required for those particular pathways are switched off. Additionally, gene expression can be altered post-transcriptionally, translationally and post-translationally.

Transcriptional regulation can be achieved by epigenetic modification of the chromatin surrounding the target genes. Chromatin is susceptible to differing alterations ranging from acetylation, deacetylation, phosphorylation, methylation, ubiquitination, sumoylation and ADP-ribosylation. All of these modifications lead to a rearrangement in the DNA-histone interactions and thereby make the gene regulatory sites either more or less accessible, hence affecting transcription. Epigenetic changes are stably inherited through repeated cell divisions and are reversible in nature. These properties of epigenetic regulation enable it to assume an irreplaceable role during developmental process.

This study was conducted to identify the regulatory roles of AtRPD3B, one member of the RPD3-type histone deacetylases in *Arabidopsis thaliana*. We were able to

demonstrate that AtRPD3B, which serves as an epigenetic regulator, plays vital functions in the jasmonic acid (JA), senescence and flowering signal networks.

Molecular Mechanisms of Histone Deacetylase Action and Recruitment

Genomic DNA exists as a protein bound complex within the cell. The proteins in question are the histone molecules that are basic in nature. The N-terminal tails of the histone sequences are highly charged with basic residues such as lysine and arginine. A nucleosome is comprised of ~146 bp negatively charged DNA wrapped around a histone octamer containing of two molecules each of H2A, H2B, H3 and H4. DNA-histone interactions mask the gene regulatory sites, thus blocking the transcriptional activation of the embedded genes. To overcome the difficulty, core histones can exist in multiple alternative states of acetylation, methylation, phosphorylation, ubiquitination or ADP-ribosylation, which determines their charge and consequently increases or decreases their affinity for DNA. Histone deacetylases (HDACs) function to remove acetyl groups from the histone tails, thereby increasing the positive charge of the histones and enhancing their affinity for DNA.

The identification of the first mammalian HDAC revealed the existence of a family of proteins in higher eukaryotes related to the yeast proteins RPD3 and HDA1. These two protein subgroup with histone deacetylase activity were identified in mammals as the classical HDAC group which can be divided into two different the phylogenetic classes, namely class I and II (Bjerling et al, 2002) and the SIR2 family of NAD-dependent HDACs. The mammalian class I HDACs, comprised of HDAC1, 2, 3, and 8, are most closely related to the yeast (Saccharomyces *cerevisiae*) transcriptional regulator

RPD3 (the first class in yeast consists of Rpd3p, Hos1p, and Hos2p). The mammalian class II HDACs, comprised of HDAC4, 5, 6, 7, 9, and 10, share domains with similarity to yeast HDA1 (the second class in yeast contains Hda1p). These HDACs, taken together with the prokaryotic enzymes acetylspermine deacetylase (ASD) and acetoin utilization protein (acuC), constitute a deacetylase superfamily (Leipe and Landsman**.,** 1997). The homology between RPD3 and HDA1 is localized to a region that is homologous to the prokaryotic enzymes. This region of histone deacetylases has been termed the acuC/APH (acetylpolyamine aminohydrolases) homology domain (Khochbin and Wolffe, 1997). Therefore, it was suggested that the deacetylase activity is associated with this portion of the proteins. This domain covers two thirds of the sequence from the NH_2 terminal and contains stretches of absolutely conserved amino acids. Open reading frames (ORFs) with high sequence homology to the acuC/APH domain were found in invertebrates, *Caenorhabditis elegans* and *Drosophila* (dHDAC1 and dHDAC3) and in vertebrates, amphibians, birds and mammals (Leipe and Landsman**,** 1997; Khochbin and Wolffe, 1997). HDAC functionality has been reported in *Xenopus* also (Wong and Wolffe, 1995).

In mammals, HDACs are known to be recruited in complexes with sequence-specific regulatory factors such as Sin3, NuRD, and CoREST (Ahringer, 2000; You et al, 2001). Additionally, they can also be recruited in response to high methylation in association with methyl-DNA binding domains (MBD) containing proteins such as MeCP2 and MBD2 (Bird and Wolffe, 1999). HDACs themselves are under regulation by subcellular compartmentalization, postranscriptional modification and interacting proteins (Yang and Seto, 2003). HDACs can directly interact with a DNA binding protein that specifically associates with a set of promoters. For instance, HDAC1 interacts

directly with the transcription factor YY1 (Yang et al, 1996) and HDAC4, 5, 7, and 9 bind to the MEF family of transcription factors. Additionally, HDACs can be sequestered into extranuclear compartments by phosphorylation and can be held in inactive states until required (Grozinger and Schreiber, 2000). Therefore, HDACs target genes that they regulate via a multitude of combinatorial processes and serve to alter their expression.

In plants, HDACs were first reported by Sendra *et al* (1988) and subsequent research has led to the characterization of 16 potentially functional HDACs in *Arabidopsis thaliana*. These 16 HDACs have been classified into three families: the RPD3/HDA1-superfamily, the SIR2-like family, and the plant-specific HD2-like HDACs originally identified in maize as acidic nucleolar phosphoproteins (Lusser et al, 1997). The RPD3/HDA1 superfamily consists of a collection of member proteins organized into different groups. HDA6 (AtRPD3B), HDA7, HDA9, HDA10 and HDA19 (AtRPD3A) comprise the first group of RPD3 type proteins (Wu et al, 2000b; Murfett et al, 2001; Tian and Chen, 2001; Pandey et al, 2002). HDA5, HDA15 and HDA18 constitute the second group and HDA2 forms the third group. The SIR2 family is represented by HDA12 (SRT1) and HDA16 (SRT2) in *Arabidopsis* (Pandey et al, 2002). A novel class of histone deacetylases was reported that shared no homology with the known eukaryotic HDAC sequences rather was similar to non-HDAC proteins such as FKBPs and RNA-binding proteins from yeasts and insects, respectively (Lusser et al, 1997). These proteins which are called the HD2-type HDACs are now considered the third class of HDACs unique to plants

What is the Functional Significance of HDACs?

Histone deacetylases assume a predominant role in several important regulatory processes in most model systems examined. For instance, in yeast, deletion of *RPD3* has been shown to repress (‘‘silence’’) reporter genes expression when inserted near telomeres (Bernstein et al, 2000). The gene expression profiles demonstrate that 40% of endogenous genes located within 20 kb of a telomere are down-regulated if *RPD3* has been deleted. Rpd3p also appears to activate telomeric genes repressed by the silent information regulator (SIR) proteins directly (Bernstein et al, 2000). In mammals, some class I HDACs participate in the control of cell cycle progression by cooperating with the co-repressor Rb (Brehm, 1998; Ferreira et al, 1998). HDACs are involved in homeotic gene silencing in *Drosophila* (Chang, 2001) and regulate post-embryonic organ transformations in *Xenopus* (Sachs et al, 2001). Genome-wide analysis in yeast revealed that RPD3-type HDACs affect the acetylation of genes in virtually all cellular pathways (Robyr et al, 2002), but preferentially associate with promoters that direct high transcriptional activity such as ribosomal protein genes or rRNA genes (Kurdistani et al, 2002). It is believed that HDACs mediate their activities by forming complexes with other heterochromatinic proteins such as polycomb and DNA methyltransferase proteins. Additionally, the SIR2 family is known to play roles in the repression of the silent mating type loci (Imai et al, 2000; Guarente, 2000), repression of rRNA gene recombination, repression of protein-coding genes inserted near telomeres or within rRNA gene arrays and cellular ageing (Guarente and Kenyon, 2000). In plants, the roles of HDACs are not so well defined. Nevertheless, there are several reports delineating an essential function for HDACs in plant development, stress tolerance and pathogen resistance. Mutants in an

Arabidopsis RPD3-like HDAC gene, *AtRPD3B*, were found in two independent mutant screens based on their effects on specific transgene expression (Murfett et al, 2001). Mutations in *AtRPD3B* affected transgene expression, DNA methylation, and regulation of rRNA genes (Murfett et al, 2001; Aufsatz et al, 2002; Probst et al, 2004). Additionally, a range of developmental abnormalities including suppression of apical dominance, reduced sterility, and delayed flowering were observed in plants expressing antisense *AtRPD3A* construct as well as *AtRPD3A* T-DNA insertion mutanst (Wu et al, 2000; Tian et al, 2001; Tian et al, 2003). It has also been suggested that AtRPD3A is a global regulator for general deacetylation, whereas AtRPD3B is responsible for a more specific function. Interestingly, the HD2-type HDACs seem to be involved in embryo maturation and hormone response pathways in plants (Wu et al, 2000a; Wu et al, 2003; Zhou et al, 2004).

Leaf Senescence Programming in *Arabidopsis*

In monocarpic plants, reproductive development often controls senescence of the whole plant with a dramatic effect on leaf senescence. In other words, reproductive growth reciprocally replaces vegetative growth (Nooden and Penny, 2001). *Arabidopsis* is an exception to this pattern, which is followed by most other monocarpic plants in the Brassicaceae (Hensel et al, 1993). It was proposed that leaf senescence in *Arabidopsis* is an age regulated phenomenon rather than a correlatively controlled program. There are internally programmed sensors for leaf ageing that initiate programmed cell death and apical arrest after the leaf has reached a certain age, irrespective of reproductive

efficiency (Hensel et al, 1993; Nooden and Penney, 2001). This special characteristic of *Arabidopsis* makes it a very good model for leaf senescence study.

Leaf senescence is the sequence of degradative processes leading to the remobilization of nutrients and eventual leaf death. It is not a chaotic breakdown, but an orderly loss of normal cell functions under genetic control of the nucleus. The senescence process is highly regulated, involving photosynthetic decline, protein degradation, lipid peroxidation, and chlorophyll degradation (Smart, 1994). The most conspicuous phenotypic change is the yellowing of leaves that is caused by the preferential breakdown of chlorophyll and chloroplasts (Gut et al, 1987). The loss of the photosynthetic pigment chlorophyll is accompanied by the breakdown of the structural integrity of the chloroplast, which leads to attenuation of energy-requiring anabolic events such as protein synthesis. Although senescence is a degenerative process, it requires de novo synthesis of specific proteins and is a genetically programmed event (Woo et al, 2001). Extensive molecular studies have indicated that leaf senescence is accompanied by decreased expression of the photosynthesis associated genes (PAGs) and increased expression of senescence-associated genes (SAGs) (Hensel et al, 1993). Some PAGs, with reduced expression during senescence, include *rbc*S (small subunit of Rubisco) and *cab* (chlorophyll *a/b*-binding protein) (Bate et al, 1991). On the other hand, identified senescence-induced genes, or SAGs, encode proteases, RNases, Gln synthetase, metallothioneins, protease regulators, ACC oxidase, lipases, glyoxylate cycle enzymes, catalase, endoxyloglucan transferase, pathogenesis-related proteins, ATP sulfurylase, glutathione *S*-transferase, Cyt P450, and polyubiquitin (Buchanan-Wollaston, 1997; Weaver et al, 1997).

The initiation of leaf senescence primarily depends upon the age of the leaf and to a lesser extent, the reproductive maturity of the plant. External factors such as nutrient deficiency, pathogenic attack, drought, light limitation, and temperature can induce premature senescence (Smart, 1994). The changing expression patterns of the PAGs and the SAGs in response to different stimuli (external and internal) is an effective marker for investigating the extent of involvement with the different affecting stimuli (Oh et al, 1996; Chung et al, 1997; Park et al, 1998; Weaver et al, 1998). Studies conducted with hormones such as ABA, ethylene, cytokinin, methyl jasmonate, wounding, dehydration, and dark treatment have shown that these genes are differentially regulated, suggesting that there are multiple signaling pathways leading to their induction (Gan and Amasino, 1997; Park et al, 1998; Weaver et al, 1998).

Recent genetic studies in *Arabidopsis* indicate that regulated protein degradation is required to control leaf senescence. ORE9 has been identified as a protein containing an F-box motif; such proteins are usually members of the ubiquitin E3 ligase complex (Woo et al, 2002). The SCF (SKP1, Cullin, F-box) complexes are known to ubiquitinate specific target substrates and recruit them for proteolysis (Patton et al, 1998). Thus, ORE9 might limit leaf longevity by removing target proteins that are required to delay the leaf senescence program in *Arabidopsis* via ubiquitin-dependent proteolysis. Potential targets might include key negative receptors of SAG. The model proposed by Lim et al 2003 extrapolates that senescence inducing factors somehow activate kinases which phosphorylate anti-senescence proteins such as self-maintainence proteins or senescence repressors. The phosphorylation of these proteins can then act as a tag that is recognized by ORE9, which can then recruit these proteins for degradation.

Telomeres exert several important functions throughout the cell cycle and there are some indications that they serve as a molecular clock to control lifespan in animal cells (Schaetzlein et al, 2004; Askree et al, 2004). Whether they have a fixed structure over the life span of an organism or structural changes are associated to specific developmental stages is still an open question. Therefore, chromatin structure and DNA/protein composition of the telomeres and their changes during the onset of leaf senescence of *Arabidopsis thaliana* are very pertinent areas of research currently. It would be interesting to know if epigenetic modifications can regulate a highly programmed process such as senescence.

Jasmonic Acid Signaling Networks in *Arabidopsis*

Jasmonic acid (JA) is the terminal product of the octadecanoid pathway and several intermediates in the pathway for JA biosynthesis are biologically active. JA is involved in wide variety of physiological processes in plants including fruit ripening, production of viable pollen, root growth, tendril coiling, plant response to wounding and abiotic stress, and defenses against insects and pathogens (Creelman and Mullet, 1997). *Arabidopsis* mutants defective in JA biosynthesis or perception are deficient in defense responses and are male sterile (Feys et al., 1994; McConn and Browse, 1996; Vijayan et al, 1998). The JA signal pathway integrates several signal transduction events: firstly, the signal for primary wound and stress is perceived and transduced locally and systemically; subsequently, this signal is recognized which leads to the induction of JA biosynthesis; once JA is perceived by the system, there is a dramatic induction of responses that finally

integrate JA signaling with outputs from the SA, ethylene, and other signaling pathways (Turmer and Devoto, 2002).

In an effort to identify membrane-spanning receptors for JA, two atypical proteins were identified, namely, COI1 (coronatine insensitive) and JAR1 (JA resistant) (Ellis and Turner, 2001). COI1 was found to be an F-box protein (Xie et al, 1998), whereas JAR1 was similar to an auxin-induced GH3 gene product from soybean. Neither of these two proteins showed homology to previously described plant receptor proteins (Gilroy and Trewavas, 2001) suggesting that either there was a genetic redundancy in the types of JA receptors, or that *COI1* and *JAR1* functioned in JA perception.

The *coi1* mutants were observed to be unresponsive to growth inhibition by MeJA, male sterile, and did not express JA-regulated genes for *vegetative storage protein* (*VSP*) (Benedetti et al., 1995), *thionin2.1* (*Thi2.1*), and the *plant defensin 1.2* (*PDF1.2*). Additionally, these mutants were found to be vulnerable to insect herbivory and pathogens (McConn et al., 1997; Thomma et al., 1998). It was demonstrated that, although *JAR1* is required for JA-dependent defenses, it is not essential for stamen and pollen development. Rather, stamen and pollen development were shown to require only *COI1*, indicating two pathways for JA perception: one for plant defense requiring both *JAR1* and *COI1* and another for pollen development requiring only *COI1*. Therefore, COI1 was implicated to participate in two different JA response pathways. A 66-kD protein was characterized to be encoded by the COI1 gene and the protein sequence contained an N-terminal F-box motif and a leucine-rich repeat domain (Xie et al., 1998). F-box proteins occur in the eukaryote kingdom in organisms from yeast to man, and function as receptors that recruit regulatory proteins as substrates for ubiquitin-mediated

destruction (Turner et al, 2002). F-box proteins associate with cullin and Skp1 proteins to form an E3 ubiquitin ligase known as the SCF complex (Bai et al., 1996). COI1 was therefore expected to form a functional E3-type ubiquitin ligase in plants that would recognize key regulatory proteins and target them for regulation (Figure 1A). Consistent with that, histone deacetylase AtRPD3B and small subunit of rubisco were found to be target binding proteins of the COI-SCF complex (Devoto *et al*, 2002). This was an interesting revelation as it implied epigenetic interference by HDACs in the jasmonate signaling pathway which was seemingly the cause for their recruitment for proteolysis by COI1. Devoto and Turner (2002) proposed a model for the interaction between JA signaling and AtRPD3B. They suggested that external stimuli, lead to JA biosynthesis and concomitantly, activate phosphorylation of regulatory proteins (repressors such as AtRPD3B) by kinases. This phosphorylation of the target regulatory proteins serves as a tag for recognition by COI1 which then polyubiquitinates or monoubiquitinates the proteins. Polyubiquitination recruits the protein for proteolysis and monoubiquitination somehow serves to activate the protein (Figure 1B). Based on these findings, it would be interesting to investigate the status of JA signaling in AtRPD3B mutants which would help to clarify their relationship.

Jasmonic acid is a major player in plant defense responses as well as developmental processes such as pollen development. There are other phytohormones which participate in defense responses, which summons for cross-talk and networking between JA and other hormone pathways. Cross-talk has been examined between JA, salicylic acid (SA) and ethylene (ET) signal pathways (Ellis et al, 2002). In JA biosynthesis deficient mutants, SA was found to accumulate and the SA induced

pathogenesis related gene (*PR1*) was upregulated (Seo et al, 1995). Conversely, enhanced JA perception and response suppress SA biosynthesis (Niki et al, 1998). Additionally, the *cev1* (constitutive expression of *VSP*) mutant which has enhanced JA signaling, demonstrated a downregulation of JA responsive *PDF1.2* and upregulation of *PR1* upon treatment with SA (Ellis et al, 2002). Therefore, JA and SA share an antagonistic relationship. On the other hand JA and ET have been shown to have a synergistic interaction with each other (Figure 2). The JA responsive *coi1* and *cev1* double mutant *coi1/cev1* demonstrated lack of *PDF1.2* and *Thi2.1* expression. The ethylene responsive *etr1* and *cev1* double mutant *etr1/cev1* had the same phenotype as the *coi1/cev1* double mutant with absence of *PDF1.2* expression (Ellis and Turner, 2001), implicating positive interaction between the JA and ET signaling pathways. At the same time, constitutive expression of JA responsive *Thi2.1* in the *etr1/cev1* mutant was indicative of the negative aspect of JA and ET signal networking. The JA and ET pathways are thought to converge at ethylene responsive factor1 (*ERF1*), as the overexpression of *ERF1* was able to rescue *coi1* and ethylene insensitive 2 (*ein2*) mutant phenotypes by restoring *PR* gene expression (Lorenzo et al, 2003). Thereby, JA works in conjunction sometimes and antagonistically in other cases with other hormone networks to expedite plant defense responses. It would be exciting to understand whether transcription regulatory factors such as histone deacetylases can function in these converging pathways to regulate their activities.

The involvement of JA in the leaf senescence programs has been investigated by He et al (2002) who demonstrated that most JA biosynthetic genes were upregulated during leaf senescence. It was shown that JA was unable to induce senescence in the JA response mutant *coi1* indicating that full JA perception and signal transduction is

essential for senescence progression. Deletion of yeast class I HDACs, *RPD3* gene led to prolonging the life-span of the yeasts by loss of rDNA silencing (Kim et al, 1999). Therefore, it was interesting to investigate whether loss of class I HDACs from *Arabidopsis* would have any effect on the life-span of the plant. Additionally, it was also established that AtRPD3B is target substrate of the JA response protein COI1 containing SCF complex (Devoto, 2002), implicating the relevance of HDACs in JA signal transduction. Further examination of the biological role of HDACs in the JA signaling will reveal whether the HDACs might participate in senescence programs via integrating the JA signal pathway.

Flowering Time Controls

Recognition of favorable environmental conditions and integration of that information with endogenous developmental cues is essential for the success of sexual reproduction in plants. Flowering in plants involves the transition from a vegetative meristem producing leaves and stems into a floral meristem producing flowers (Koornneef et al, 1998b; Simpson et al, 1999; Reeves and Coupland, 2000; Samach and Coupland, 2000; Araki, 2001). The regulation of these events occurs via a complex network of genetic pathways consisting of two pathways responsive to the environment (long-day and vernalization pathways) and two pathways independent of environmental cues (autonomous and GA pathways). To understand the genetic regulation of flowering, it is important to know that there are two sets of genes that are instrumental in this process (Blazquez and Weigel, 2000): the flowering-time genes and the floral-identity genes. The flowering-time genes assure that flowering occurs at the right time in response

to environmental and internal stimuli and in turn transmit the signals to the floral-identity genes to develop the floral organs (Araki, 2001).

The floral-identity genes can be divided into two sub-groups: the meristem identity genes, which specify inflorescence and floral meristem identity, and the floral organ identity genes, which define the identities of the organs in the flower (Coen 1991; Coen and Meyerowitz, 1991). *APETALA2* (*AP2*), *APETALA3* (*AP3*), *PISTILLATA* (*PI*), and *AGAMOUS* (*AG*) genes regulate floral organ identity in the whorled *Arabidopsis* flower. LEAFY (*LFY*), *CAULJFLOWR* (*CAL*) and *APETALA1* (*APl*) represent *Arabidopsis* meristem identity genes. In *Arabidopsis* plants ectopically expressing *LFY* or *AP1*, lateral meristems that normally would be shoots are converted into axillary flowers (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). *AG* is required to maintain floral meristem identity during reproductive growth, even in the presence of *LFY* and *AP1* (Mizukami and Ma, 1997). Most of these genes encode MADS-box (MCM1-Agamous-Deficiens-SRF) containing regulatory proteins in which this domain acts as a sequence-specific DNA-binding moiety. The MADS-box genes have been shown to mediate between meristem specification and organ identity functions (Purugganan et al, 1995) and perceive signals from upstream flowering-time pathways.

Long-day pathway (environmental response)

This genetic pathway was identified on the basis of the late flowering phenotype of mutants in long-day environments, which had wild-type flowering times in short-day and post-vernalization. *CONSTANS* (*CO*), *CRYPTOCHROME2/FHA* (*CRY2*), *GIGANTEA* (*GI*), flowering-time (*FT*), and *FWA* are members this long day-promoting signal pathway (Koornneef et al, 1991). The *CO* gene assumes a central position in the

other flowering pathways as it functional at a significantly downstream position whereby it controls the expression of the *FT* (long-day pathway) and the suppressor of constans (*SOC*:autonomous pathway) and *FWA* (long-day pathway) genes which are essential flowering inducing genes (Onouchi et al, 2000). The *CO* gene was identified to encode a protein with two zinc fingers loosely related to those of GATA transcription factors which might enable it to recognize target protein sequences (Putterill et al, 1995). The CO gene has been shown to mediate between the circadian clock and the flowering-time gene *FT* (Suarez-Lopez et al, 2001) as the transcript level of *CO* follows a diurnal rhythm in long days, with a broad biphasic peak between 12 and 24 h after dawn and maximum levels 16 and 24 h after dawn (Suarez-Lopez et al, 2001). Consequently, the *FT* gene also followed the same circadian rhythm. Therefore, researchers proposed that the circadian clock acts within the long-day pathway to regulate the expression of downstream *CO* and *FT* genes. It was suggested that the circadian clock-*CO*-*FT* cascade followed an external coincidence model (Thomas and Vince-Prue, 1997; Samach and Coupland, 2000; Samach and Gover, 2001). This model extrapolated that the expression of the *CO* gene follows a specific rhythm, which is light-sensitive. The expression of this gene will peak when exposed to light at a certain time in the day, which is afforded only under long-day conditions that will lead to the induction of flowering (Suarez-Lopez et al, 2001). Therefore, the clock plays a crucial role in setting the times in a day, so that at the appropriate time, the light signal and *CO* gene can interact. Importance of the clock functioning during the long-day pathway was evident in experiments where CO transcript levels were altered in clock-related mutants *LATE ELONGATED HYPOCOTL (LHY*), *GI* and *EARLY FLOWERING3* (*ELF3)* (Suarez-Lopez et al, 2001).

Vernalization pathway (environmental response)

The acceleration in flowering upon exposure to low temperatures for several weeks is known as the vernalization response. Two genetic loci were identified as essential to confer this phenotype on the winter annuals, namely, flowering locus C (*FLC*) and frigida (*FRI*) (Burn et al, 1993a; Lee and Amasino, 1993; Clarke and Dean, 1994). FLC is a MADS-box transcription factor that encodes a repressor of flowering (Michaels and Amasino, 1999b; Sheldon et al, 1999, 2000). It was shown that the abundance of *FLC* mRNA fell when the plants were exposed to cold, and that this reduction occurred progressively in a way that was consistent with the progressive effect on flowering time (Sheldon et al, 2000). The biochemical function of FRI protein was not clear but it was predicted to contain coiled-coil domains that may enable the protein to be involved in protein–protein interactions (Johanson et al, 2000). The FRI protein was proposed to act upstream of FLC as flc mutations abolished the FRI phenotype (Michaels and Amasino, 1999b; Sheldon et al, 1999). Once FLC and FRI were established as the essential flowering repression genes and it was clear that cold-treatment led to their repression and subsequent flowering, researchers shifted focus to the maintenance of the FLC/FRI repression post-cold treatment. The FLC repression was stably maintained post-vernalization, implicating epigenetic regulatory mechanisms (Burn et al, 1993b). Subsequently, methylation was identified to be a key repressor of FLC chromatin thereby maintaining post-vernalization repression (Finnegan et al, 1998). Consistent with that, vernalization2 (*VRN2*) was reported to maintain the stable repression of *FLC* (Gendall et al, 2001). Although VRN2 is a polycomb (PcG) group protein (Birve et al, 2001; Gendall

et al, 2001), it might be acting together with methylation to stably repress *FLC*. It is possible that histone deacetylases also participate in this repressive complex to regulate flowering as it is becoming evident that all the chromatin modifying agents such as HDACs, PcGs and Methylating proteins might be recruited as a complex to target genes.

Autonomous pathway (endogenous response)

This internal genetic pathway in *Arabidopsis* was characterized by mutants that were late-flowering irrespective of different photoperiods (short-day or long-day) (Martinez-Zapater and Somerville, 1990; Koornneef et al, 1991). The late-flowering mutants could be rescued by vernalization, which indicated a relationship between the autonomous pathway and *FLC* expression (Michaels and Amasino, 1999b; Sheldon et al, 1999). Consistently, the *flc* mutants were found to suppress the autonomous pathway mutant's phenotype (Michaels and Amasino, 2001). The mutants of this pathway have been characterized as *fca*, *fy*, *fpa*, *luminidependens* (*ld*), and *fve*. All of these mutants had increased levels of *FLC* expression indicating that the autonomous pathway functions by repressing *FLC*. This would also explain the ability of vernalization to rescue the mutant phenotypes. FCA and FPA have been identified to be RNA binding proteins (Macknight et al, 1997) with RNP (RNA recognition motif or consensus sequence RNA-binding domain) motifs that suggested that post-transcriptional regulation may be playing a general role in the pathway (Schomburg et al, 2001). The LD protein was found to contain a homeobox and putative nuclear localization sequences, and was predicted to encode a transcription factor (Lee et al, 1994b). It has not yet been established as to how these autonomous pathway genes serve to downregulate *FLC*, but there is evidence that

the genes of this pathway do not follow a linear cascade (Koornneef et al, 1998b). Rather, each protein has additional functions other than regulating flowering time.

Two other pathways exist for flowering-time regulation, namely, the GA pathway and the circadian pathway. All flowering pathways ultimately converge to control a downstream set of genes that lead to floral meristem and organ formation.

Common set of genes between different flowering pathways

The circadian clock acts within the long-day pathway to control *CO* gene expression, whereas the GA pathway and the long-day pathway have been shown to converge on the *LFY* promoter, rather than both pathways activating an earlier acting gene that in turn increases the expression of *LFY* (Blazquez and Weigel, 2000). On the other hand the long-day and the autonomous pathways were distinct upon their effector proteins FLC and CO activity. Downstream from there on, these two pathways converge upon the target genes for these two proteins, *SOC1* and *FT* and their subsequent target genes. This was supported by experiments conducted by Suarez-Lopez et al (2001) where they demonstrated that ectopic expression of *FLC* delays flowering, but does not affect *CO* gene expression. Complementarily, it was observed that mutations in *CO* did not affect *FLC* expression (Sheldon et al, 1999). The *SOC1* and *FT* genes are major downstream targets of FLC and CO, whereby *SOC1* is repressed by FLC and upregulated by CO (Borner et al, 2000; Lee et al, 2000; Samach et al, 2000; Michaels and Amasino, 2001). This is interesting, as *SOC1* is also an agamous like (*AGL20*) gene that may directly be involved in floral organ formation. Additionally, the *FT* gene is also repressed by FLC and activated by the CO pathway (Kardailsky et al, 1999; Kobayashi et al, 1999; Samach et al, 2000). FT was identified to be homologous to mammalian

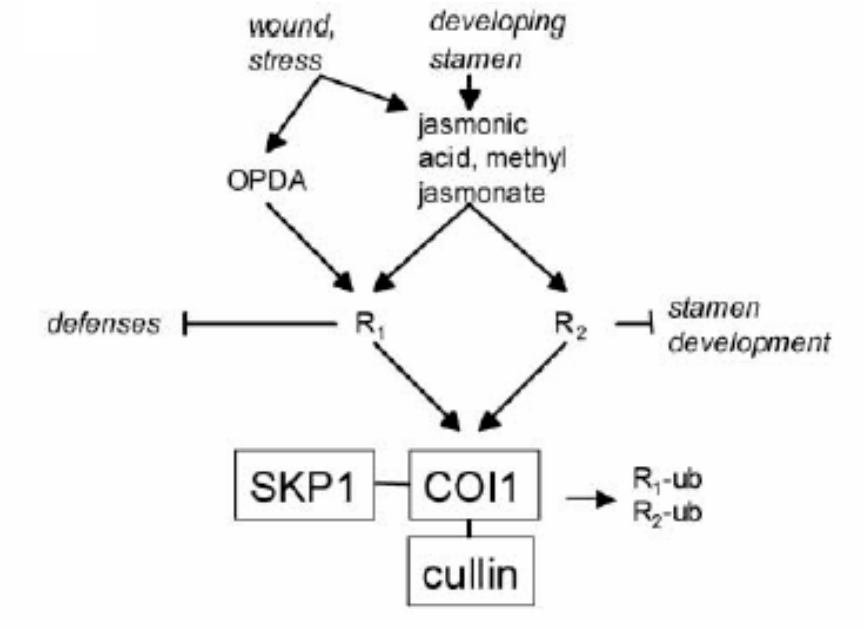
phosphatidylethanolamine binding proteins (PEBP), which were originally shown to bind phospholipids (Bradley et al, 1997). *FT* was shown to activate flowering by controlling floral identity genes such as *AP1* (Ruiz-Garcia et al, 1997). A model that illustrates the flowering pathway and their interactions is shown in Figure 3.

Epigenetic regulation of floral repression

The maintenance of post-vernalization repression of *FLC* by *VRN2* was a significant discovery for understanding floral repression genetics (Chandler et al, 1996). It brought to light the fact that epigenetic regulation plays a key role in the vegetative to flowering transition phase. VRN2, being a Drosophila polycomb group protein, Su(z)12 homolog, was able to downregulate *FLC* in a stable manner and methylation was deemed the cause for this stable repression (Burn et al, 1993b). In matter of fact, PcG proteins act in multiprotein complexes to silence chromatin by modifying histone N-terminal tails through deacetylation and methylation (Fischle and Allis, 2003). In humans and Drosophila, Su(Z)12 polycomb proteins have been shown to mediate gene silencing by a series of histone modifications leading to methylation of histone H3 at Lysine 9 (Kuzmichev, 2002). A transiently acting repressor is essential to target specific genes and this repressor could be represented by HDACs. Therefore it is possible that HDACs might also be recruited in the PcG-methylation complex to silence *FLC*. Consistent with this idea, Sung and Amasino (2004) demonstrated that *FLC* chromatin had reduced levels of acetylation during vernalization treatment. Additionally, epigenetic regulation of *FLC* by the autonomous pathway has also been reported. Sanda and Amasino (1996) identified the sixth member of the autonomous pathway, FLD. Subsequently, He et al (2003) identified FLD to be a protein containing the SWIRM domain usually found in chromatin

remodeling proteins (Aravind and Iyer, 2002). Also, the human homolog of this protein was identified by them to be a component of the human HDAC1/2 complex (Humphrey et al, 2001; Hakimi et al, 2003). Increased acetylation levels of histone H4 in *FLC* chromatin in the *fld* mutants indicated that FLD, like autonomous pathway member FVE, also achieves FLC repression by deacetylation. Additionally, the mechanism by which FLC maintains repression of target genes is of particular interest. It would be interesting to examine if there is an epigenetic aspect to FLC mediated repression.

A.


wound, stress
developing stamen
jasmonic acid, methyl jasmonate
OPDA
defenses
R1
R2
stamen development
SKP1
COI1
cullin
R1-ub
R2-ub

B.

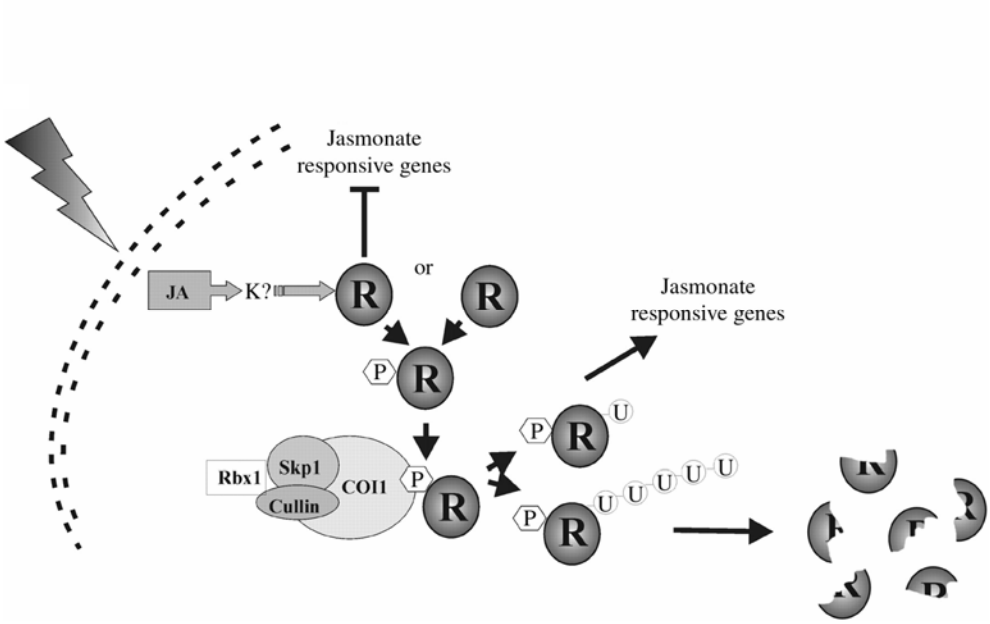

Jasmonate responsive genes
JA
K?
R
or
R
P
R
Jasmonate responsive genes
Rbx1
Skp1
Cullin
COI1
P
R
P
R
U
P
R
U
U
U
U
U

Figure 1. Integration of environmental and endogenous stimuli to activate COI1-E3 ubiquitin ligase complex mediated activities.

A. Model for how COI1 may regulate JA-dependent defense responses and JA-dependent stamen and pollen development in *Arabidopsis*, through the E3 ubiquitin ligase dependent modification of hypothetical repressors R1 and R2 of these two processes (Devoto *et al*, 2002).

B. COI1, Skp1, AtCUL1 (Cullin) and AtRbx1 (Rbx1), form an SCF^{COI1} ubiquitin ligase complex (depicted here are the only components identified so far) (Devoto and Turner, 2003). Here a signal activates synthesis of JA and phosphorylation (P) of a target acting as negative regulator (R) of jasmonate responsive genes, which now binds COI1. The ubiquitinated protein is destroyed in the proteasome. Alternatively, the SCFCOI1 ubiquitin ligase complex might activate the regulator via monoubiquitination. K, Kinase; U, ubiquitin.

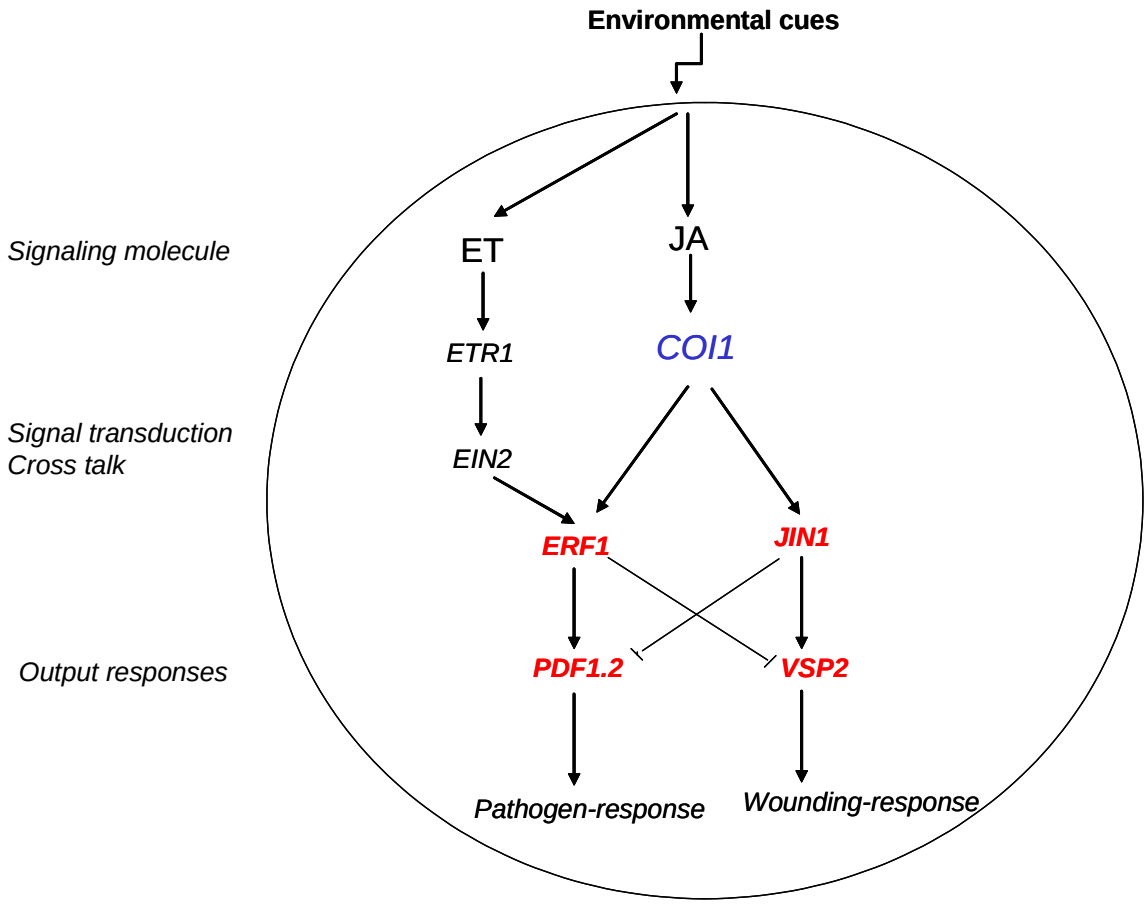


Figure 2. Model of the jasmonate (JA) and ethylene signaling pathway.

Different types of stresses, such as wounding or pathogen infection, induce the synthesis and subsequent activation of the JA and ethylene pathways. JA, via *COI1*, and ethylene, via *ETR1* and *EIN2*, act synergistically and in and ERF1-dependent manner to induce the expression of PDF1.2. A wound signal might induce the production of JA and this will stimulate the expression of JA responsive genes, like *JIN1* and *VSP2*.

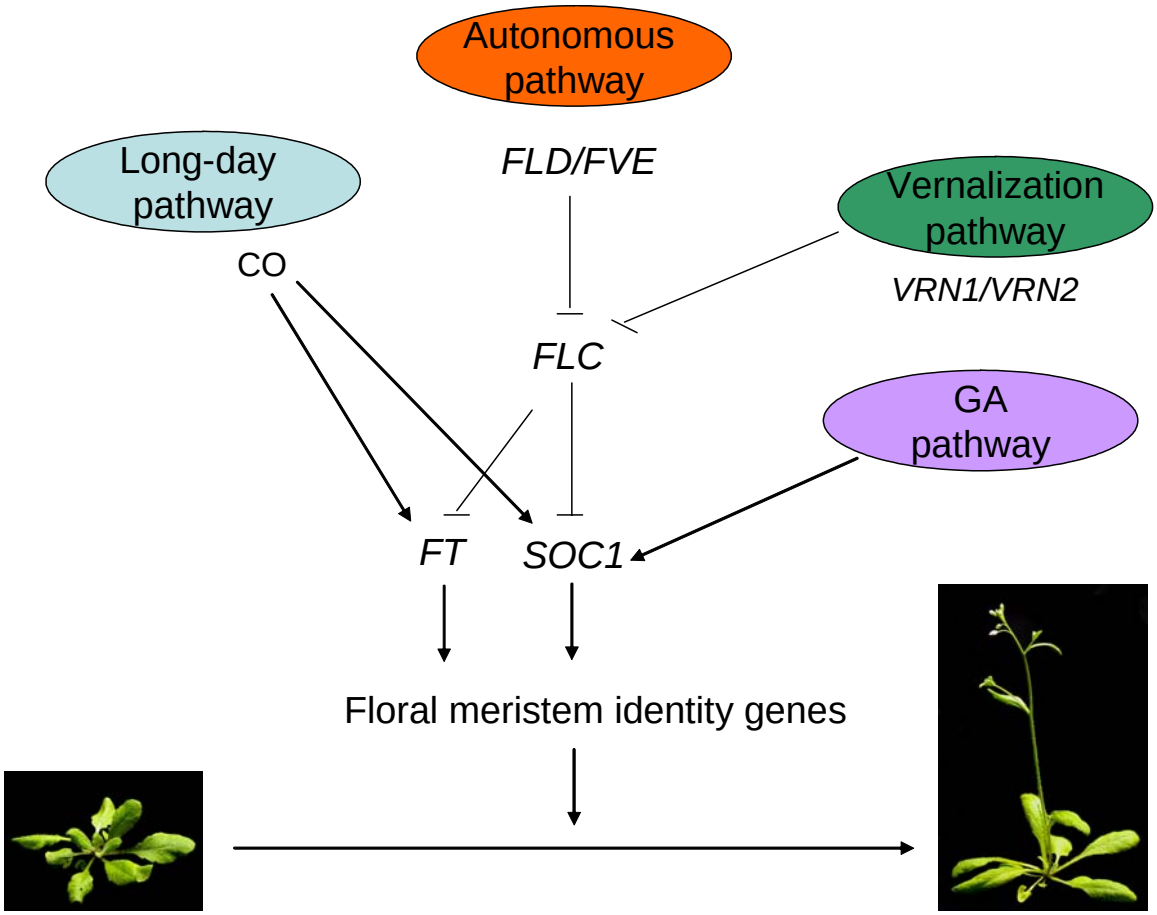


Figure 3. Schematic representation of four major genetic pathways regulating flowering time in *Arabidopsis*.

The two main pathways mediating environmental responses are the long-day and vernalization pathways. The two pathways thought to function independently of environmental cues are the autonomous pathway, which promotes flowering in all conditions, and the GA pathway, which is needed for flowering in non-inductive short-day conditions. AtRPD3B may interact with FLD and FVE to reduce FLC transcript level.

MATERIALS AND METHODS

Plant Growth Conditions and Vernalization Treatment

Arabidopsis thaliana plants were grown in a growth chamber under long-day (16 hours light/8 hours of darkness) or short-day (8 hours light/16 hours darkness) conditions. For growth under sterile conditions, seeds were surface sterilized (12-min incubation in 5% [v/v] sodium hypochlorite and a five-time rinse in sterile distilled water) and sown on half-strength MS salts (Sigma-Aldrich, St.Louis, MO) supplemented with 1% sucrose, pH 5.7, and 0.8% (w/v) agar in Petri dishes and then incubated under short- day condition at 4 °C for 2-4 days.

Vernalization was carried out by placing seeds in soil at 4^{o}C under short-day (8 hours light/16 hours darkness) conditions for 42 days. Pots were covered with plastic membrane and watered once per week. Seeds were then transferred to long-day (16 hours light/ 8 hours darkness) or short-day condition. Nonvernalized seeds were grown in soil at 4^{o}C under short-day condition for 2 days, and then transferred to the long-day or short-day condition.

Plasmid Construction

To generate *AtRPD3A* and *AtRPD3B* promoter deletion constructs, *AtRPD3A: GUS* and *AtRPD3B:GUS* constructs were used as the template for PCR. Promoter fragments of varying lengths were amplified by PCR using the primer pairs listed in Table 1. The resulting PCR products were then digested by *HindIII* and *NcoI* for *AtRPD3A* promoter deletions and *PstI* and *NcoI* for *AtRPD3B* promoter deletions and

subcloned into the pCAMBIA1381 binary vector (Cambia, Canberra, Australia). To generate the *35S:AtRPD3A-GFP* construct, AtRPD3A coding region was PCR amplified and subcloned in frame in front of the GFP of the pCAMBIA1302 vector. To generate *35S:AtRPD3B-GFP*, AtRPD3B coding region was PCR amplified to replace AtHD2A in the AtHD2A-GFP construct (Zhou et al 2003). DNA and protein sequence analysis was performed using BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/) and the Vector NTI Suite program (InforMax, Bethesda, MD).

Table 1. Primers used for making promoter deletion constructs

Constructs	Primers	Sequences	Restriction Sites
AtRPD3A:GUS	G11pro pr3a	5'-tgagccatggacgcgtttgaatga-3'	*NcoI*
	G11pro pr1	5'-aattaagcttgcttaagatggaagcatgtgc-3'	*HindIII*
-1064RPD3A:GUS	G11pro pr3a	5'-tgagccatggacgcgtttgaatga-3'	*NcoI*
	G11pro pr4s	5'-aattaagcttagatgcggatgcgcatgatg-3'	*HindIII*
-742RPD3A:GUS	G11pro pr3a	5'-tgagccatggacgcgtttgaatga-3'	*NcoI*
	G11pro pr5s	5'-aattaagcttcttggatacttgtagcctag-3'	*HindIII*
-508RPD3A:GUS	G11pro pr3a	5'-tgagccatggacgcgtttgaatga-3'	*NcoI*
	G11pro pr6s	5'-aattaagcttgttactctgcgtaagacc-3'	*HindIII*
-222RPD3A:GUS	G11pro pr3a	5'-tgagccatggacgcgtttgaatga-3'	*NcoI*
	G11pro pr7s	5'-aattaagcttctcctccgaccatttgac-3'	*HindIII*
AtRPD3B:GUS	164pro pr2	5'-gcctccatggccgtctctcactcagaatc-3'	*NcoI*
	164pro pr1	5'-atcgctgcagctgcagttgtagggataagg-3'	*PstI*
-1017RPD3B:GUS	164pro pr2	5'-gcctccatggccgtctctcactcagaatc-3'	*NcoI*
	164pro pr3	5'-atcgctgcagagctggtcaagttgtacctc-3'	*PstI*
-757RPD3B:GUS	164pro pr2	5'-gcctccatggccgtctctcactcagaatc-3'	*NcoI*
	164pro pr4	5'-atcgctgcagacggtggaaagaggacttgg-3'	*PstI*
-374RPD3B:GUS	164pro pr2	5'-gcctccatggccgtctctcactcagaatc-3'	*NcoI*
	164pro pr5	5'-atcgctgcagcccaacaacatctagttacg-3'	*PstI*
AtRPD3A-GFP	HDA19-5'gfp	5'-aattccatggatactggcggcaattc-3'	*NcoI*
	HDA19-3'gfp	5'-aattagatctgttttaggaggaaacgcctg-3'	*BglII*
AtRPD3B-GFP	HDA6-GFPpr1	5'-aatttcccgggcatgaggcagacgaaagcggca-3	*XmaI*
	HDA6-GAL4pr2	5'-aattgagctcttaagacgatggaggattcacg-3'	*SacI*

Plant Transformation and Selection

Arabidopsis thaliana (Columbia ecotype) plants were grown in a growth chamber under long-day (16 hours light/8 hour dark) at 22°C after a 3-4 days vernalization period for the seeds sown. These plants were grown for a period of 35 days or until the primary inflorescence was 5 to 15 cm tall and the secondary inflorescences were appearing at the rosette. Plant transformation plasmids were electroporated into *Agrobacterium tumefaciens* GV3101 as described by Shaw (1995) and inoculated in a 500 ml culture of LB medium containing 50 mg/L rifampicin, 25 mg/L gentamycin and the appropriate antibiotic for the construct with a 1 ml overnight starter culture at 30°C with shaking. When OD600 is > 2.0, the 500 ml culture was spun down at 3,000 rpm for 5 min and resuspended in 1 liter of infiltration medium containing 2.2 g MS salts, 50 g sucrose and 200 µl/L Silwet L-77 (Lehle Seeds, catalog # vis-01). When plants are ready to transform, the *Agrobacterium*-mediated floral dip transformation of *Arabidopsis thaliana* was performed as described by Clough and Bent (1998). T1 seeds were harvested from the fully grown mature transformed plants and dried at 25°C and germinated on sterile medium containing 40 mg/mL of kanamycin or hygromycin to select the transformants. Surviving T1 plantlets were transferred to soil to set seeds (T2).

GUS Assays

For histochemical GUS staining assays, transgenic tissue expressing promoter driven GUS was harvested and incubated in beta-glucuronide solution for a period of 12 hours at 37°C. *Arabidopsis* tissues were immersed in 1 mM 5-bromo-4-chloro-3-indolyl-glucuronic acid solution in 100 mM sodium phosphate, pH 7.0, 0.1 mM EDTA, 0.5 mM

ferricyanide, 0.5 mM ferrocyanide, and 0.1% Triton X-100, followed by washing with 70% ethanol to remove the chlorophyll (Jefferson, 1988).

For GUS activity assay of plant extracts, 200-500 mg *Arabidopsis* tissues were harvested in a 1.5 ml microcentrifuge tube and frozen in liquid nitrogen immediately and ground the tissue to a fine powder. 150 µl of GUS extraction buffer (50 mM sodium phosphate pH 7.0, 10 mM EDTA pH 8.0, 0.1% SDS, 0.1% Triton X-100) was added into each tube and tubes were stored in liquid nitrogen while processing. All the samples were centrifuged at 15,000 rpm for 10 minutes in the cold microcentrifuge tubes. Supernatant ("protein extract") was transferred into a fresh tube and kept on ice. 1 ml reaction mix containing 1mM 4-MUG (4-methylumbelliferyl beta-D-glucuronide) in GUS extraction buffer was added in each tube and prewarmed at 37°C. 10 µl protein extract was mixed with 1ml reaction mix. After exactly every 10 minutes, 100 µl reaction was transferred into the 900 µl stop regent (1M Na_2CO_3). The extracted GUS hydrolyzes the 4-MUG to the fluorescent compound 4-MU (pKa 8.2) and glucuronic acid. The reaction was stopped with sodium carbonate buffer because 4-MU exhibits maximal fluorescence at pH values above its pKa. The 4-MU standard curve was prepared by diluting the 4-MU stock to 10 mM, 250 nM and 500 nM in stop reagent (1M Na_2CO_3). The fluorescence of these solutions at excitation wavelength 365 nm and emission wavelength 455 nm were measured respectively. A GUS standard curve of fluorescence against the concentration was plot. The fluorescence of each sample was measured and the standard curve was used to calculate the amount of 4-MU per minute for each sample. The total protein concentration in each sample was determined using Bio-Red protein assay kid. A protein standard curve of fluorescence against the protein concentration was plot. The protein

concentration of each sample was calculated using protein standard curve. GUS activity was determined in (nmol 4-MU) min^{-1} (mg protein) $^{-1}$.

GFP Localization

Transgenic seeds were germinated on MS medium containing selection antibiotic. All these plates were covered with foil completely and grown in a growth chamber at 22°C for about 5 weeks. Protoplasts were isolated from 5-week-old transgenic *Arabidopsis* seedlings as described by Weigel and Glazebrook (2002). The entire transgenic seedlings were soaked in fresh made enzyme solution (0.25% Maceroenzyme R10 and 1% Cellulase R10) and kept at room temperature for 10 minutes. Subsequently, the tissue-enzyme mixture was subjected to vacuum gently for thirty minutes. The solution was then incubated at room-temperature and shaken for 90 minutes at 40 rpm and then at 90 rpm for 5 minutes. After that, the solution was filtered through a 70 µm nylon mesh and protoplasts were ready for use. The fluorescence photographs of protoplasts were taken using an Olympus florescent microscope (Tokyo, Japan) fitted with fluorescein isothiocyanate filters (excitation filter, 450 to 490 nm; emission filter, 520 nm; and dichroic mirror, 510 nm).

DNA Extraction

For DNA extraction from *Arabidopsis*, plant tissue was ground in liquid nitrogen with a mortar and pestle. For 0.2 g tissue, 0.6 ml of plant DNAzol (Invitrogen Carlsbad, CA, USA) was added and the plant tissues were further ground. Once the tissues melted, they were collected in microcentrifuge tubes and incubated at room temperature for 5 minutes. The mixture was centrifuged at 12000 g for 10 minutes. Aqueous phase was

transferred into a fresh tube. Subsequently, 600 µl of 100% chloroform (Fisher Scientific, Fairlawn, NJ, USA) was added to the plant extract supernatant and mixed. This mixture was incubated for 10 minutes at room temperature and subsequently spun for 10 minutes. The supernatant was retrieved and mixed with 1 ml of 100% ethanol (Fisher Scientific) and incubated for 5 minutes at room temperature. The mixture was spun for 10 minutes to pellet the DNA. The pellet was then washed with Plant DNAzol Wash (0.75 ml 100% ethanol + 1 ml of DNAzol) , then with 70% ethanol and air dried. The DNA was dissolved in sterile distilled water and stored at 4°C.

Plant Hormone Sensitivity Assay

Hormonal sensitivity was tested by examining seedling responses to different concentrations of JA. Wild type and mutant seedlings were grown as follows: Seed were surface-sterilized for 12 minutes in 5% [v/v] sodium hypochlorite followed by a five-time rinse in sterile distilled water and sown on half-strength MS salts (Sigma-Aldrich, St.Louis, MO) supplemented with 1% sucrose, pH 5.7, and 0.8% (w/v) agar in Petri dishes with or without the addition of JA (1, 5, 10, 25, 30, 50, 75 µM). Seeds plated on MS medium with or without JA were incubated at 4°C for 3 days and all the plates were then incubated vertically under long-day conditions for about 7 days.

Measurement of Chlorophyll Content and Photochemical Efficiency

From about 15 DAE (days after leaf emergence, DAE) onwards, the 6th rosette leaf that was fully grown was chosen for photochemical efficiency of photosystem II (PSII) measurement and chlorophyll extraction. At least 10 plants were used for one-time point measurements.

The photochemical efficiency of photosystem II (PSII) was deduced from the characteristics of chlorophyll fluorescence (Oh et al., 1997) using a portable plant efficiency analyzer (Hansatech Instruments, Morfolk, England). The ratio of maximum variable fluorescence to maximum yield of fluorescence, which corresponds to the potential quantum yield of the photochemical reactions of PSII, was used as the measure of the photochemical efficiency of PSII (John et al., 1995; Raggi, 1995; Oh et al., 1997). Fresh leaf will be put into portable plant efficiency analyzer and the ratio of Fv/Fm (maximum variable fluorescence to maximum yield of fluorescence) will be used as the measure of photochemical efficiency of PSII.

After the measurement of photochemical efficiency of PSII, the same leaf was then used for further chlorophyll content assay. Leaves were cut at the same area using leaf puncher and then stored into liquid nitrogen. Subsequently, the frozen leaf tissue was ground in liquid N using mortar and pestle. 4 ml of 80% acetone was added after liquid N evaporated and mixed. Liquid was then transferred into 15 ml centrifuge tube and centrifuged at 13,000 rpm for 3 minutes. 3 ml supernatant was transformed into a glass cuvette. The spectrophotometer was zeroed at 750 nm and read at 663.6 nm and 646.6 nm, respectively. Chlorophyll concentration of leaf was measured every five days. The

concentration of chlorophyll a and b was caculated using the method of (Porra et al, 1989):

$Chla = 13.71A^{663.6} - 2.85A^{646.6}$

$Chlb = 22.39A^{646.6} - 5.42A^{663.6}$

$Chla+b = 19.54A^{646.6} + 8.29A^{663.6}$ (unit: nmol/ml).

Total RNA Extraction

Total cellular RNA was isolated from plant tissue ground in liquid nitrogen. TRIzol Reagent (Invitrogen) was added and the tissue was further ground. Quantity of TRIzol depends on the amount of sample. Usually, for 1-2 g of tissue, 1 ml of TRIzol can be used. If there is larger quantity of tissue, up to 2 ml of reagent can be added. Once the tissue melted, it was collected into a 1.5 ml centrifuge tube and stored on ice and then incubated together at room temperature for 5 minutes. Subsequently, 200 µl of 100% chloroform (Fisher Scientific) was added to the plant extract and mixed. This mixture was incubated for 15 minutes at room temperature and subsequently spun at 12,000 g for 15 minutes at 4ºC. The supernatant was retrieved and mixed with 0.5 ml of isopropanol (Fisher Scientific) and incubated for 10 minutes at room temperature. The mixture was spun at 12,000 g for 10 minutes to pellet the RNA at 4ºC. The pellet was then washed with 70% ethanol and air-dried. Spin 5 minutes at 75,000 g to pellet RNA and the RNA was then dissolved in 25-50 µl sterile DEPC treated water and samples were strored in -80 ºC for further usage.

RT-PCR analysis

The isolated RNA was digested first with DNase to remove genomic DNA. 10 units DNase and 5X buffer were added into 30 µl RNA sample. The mixture was then incubated at 37°C for 30 minutes. Subsequently, the mixtue was purified using RNA Clean-up Kit-5™ (Zymo Research). One microgram of total RNA was used for the first-strand cDNA synthesis after incubation at 65°C for 10 min as described by Weigel and Glazebrook, 2002). cDNA was synthesized in a volume of 20 µl that contained MoMLV RT buffer (Promega, Madison, WI, USA), 10 mM dithiothreitol, 1.5 µM poly (dT) primer, 0.5 mM dNTPs, 2 units of MoMLV reverse transcriptase at 37°C for 1 hour. All PCR reactions were performed with 0.5 unit of *Taq* polymerase (PGC Scientific, Gaithersburg, MD, USA), the buffer provided by the supplier, 0.2 µM dNTPs, and a pair of primers (0.1 µM each) in a final volume of 20 µl. PCR parameters differed for each gene: thermocycling conditions were 94°C for 2 min followed by 25-40 cycles of 94°C for 1 min, 50-70°C for 1 min, and 72°C for 2 min, with a final polymerization step at 72°C for 10 min. The primers used for RT-PCR are listed in Table II. *Ubiquitin 10 (UBQ10)* served as internal control.

Table 2. Primers Used for RT-PCR

Primers	Sequences	Annealing Temperature	Cycle #
AtRPD3B-RT-1	5'-tagagccggacaacaaactc-3'	57°C	40
AtRPD3B-RT-2	5'-ttcacgtctgggctctgggtt-3'		
SAG12-RT- 1	5'-cagctgcggatgttgttg-3'	54°C	30
SAG12-RT- 2	5'-ccactttctccccattttg-3'		
SEN4-RT-1	5'-tcttcttcacgactcttctc-3'	58°C	28
SEN4-RT-2	5'-ttgcccaatcgtctgcgttc-3'		
RPS17-RT-1	5'-atgataacgtcgtccctaac-3'	57°C	30
RPS17 3'	5'-gctgagactccaagggaagg-3'		
VSP2 rt1	5'-ttctatgccaaaggacttgc-3'	58°C	26
VSP2 rt2	5'-gagtggatttgggagcttaa-3'		
ERF1 rt1	5'-aagctgctttctcgatgaga-3'	54°C	50
ERF1 rt2	5'-ttctccgtctcatcgagtgt-3'		
PDF1.2 rt1	5'-gttctctttgctgctttcgac-3'	56°C	28
PDF1.2 rt2	5'-ccatgtttggctccttcaag-3'		
JIN1 rt1	5'-tcggtgacgcaatcgcttac-3'	54°C	27
JIN1 rt2	5'-cttgctctgagctgttcttg-3'		
FLC -RT-1	5'-ttagtatctccggcgacttgaacccaaacc-3'	50°C	34
FLC- RT-2	5'-agattctcaacaagcttcaacatgagttcg-3'		
SOC1-RT-1	5'-aggatcgagtcagcaccaaa-3'	58°C	30
SOC1-RT-2	5'-ggtaacccaatgaacaattgc-3'		
UBQ1	5'-gatctttgccggaaaacaattggaggatggt-3'	68°C	30
UBQ2	5'-cgacttgtcattagaaagaaagagataacagg-3'		

Protein Gel Blot Analysis

For nuclear isolation, 500 mg of *Arabidopsis* seedling tissues were homogenized in 1 mL of Honda buffer (2.5% Ficoll 400, 5% dextran T40, 0.4 M sucrose, 25 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM *b*-mercaptoethenol, 100mg/mL of phenylmethylsulfonyl fluoride, 0.5mg/mL of antipain, and 0.5 mg/mL of leupeptin) and filtered through a 62-μm nylon mesh (Weigel and Glazebrook, 2002). Then, 0.5% Triton X-100 was added to the extract, which was incubated for 15 min on ice and centrifuged at 1500g for 5 min. The pellet was washed with Honda buffer containing 0.1% Triton X-100, gently resuspended in 1 ml of Honda buffer, and centrifuged at 100g for 5 min to pellet starch and cell debris. The supernatant was transferred to a microcentrifuge tube and centrifuged at 1800g for 5 min to pellet the nuclear. The nuclear extractwas suspended in 200 μl of 5X SDS-PAGE loading buffer (0.2 M Tris-HCl, pH 6.8, 25% SDS, 25% glycerol, and 12.5% 2-mercaptoethanol). The protein samples were loaded on 15% polyacrylamide gel and blotted onto a nitrocellulose membrane. The membrane was blocked in PBS containing 3% dry milk for 60 min and then incubated with 0.01 to 0.05 μg/ml of antiacetyl-histone H3 (catalog no. 06-599; Upstate, Charlottesville, VA) for 2 h at room temperature. After washing, the primary antibody was detected with secondary anti-rabbit horseradish peroxidase-coupled antibody (Amersham, Buckinghamshire, UK) at room temperature for 45 min. Visualization was achieved using the ECL system (Amersham).

RESULTS

Expression of AtRPD3A and AtRPD3B

1. Analysis of AtRPD3A:GUS and AtRPD3B:GUS expression

To examine the spatial expression profile of *AtRPD3A* and *AtRPD3B*, we constructed beta-glucuronidase (GUS) reporter gene fusions, *AtRPD3A:GUS* and *AtRPD3B:GUS*. 1378 bp of the *AtRPD3A* promoter and 1357 bp of the *AtRPD3B* promoter were used for making the fusions. These two constructs were stably transformed into *Arabidopsis* using *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). GUS activity was examined by histochemical chemical GUS assay (Jefferson, 1988) and the quantitative GUS assay (Gallagher, 1992). 2-week-old transgenic seedlings as well as adult transgenic plants were immersed in GUS staining buffer overnight at 37°C and blue color was recorded. High levels of GUS expression were observed in all parts of both *AtRPD3A:GUS* and *AtRPD3B:GUS* seedlings (Figure 4A and B). In adult plants, *AtRPD3A* promoter drove higher levels of GUS expression in all organs examined, including leaves, stems, flowers and siliques. Additionally, the GUS activity was detected in all parts of the flowers, including the sepals, petals, pistil, stamens and seeds in *AtRPD3A:GUS* plants (Figure 4A). In contrast, *AtRPD3B:GUS* showed relatively weaker expression in the leaves, stems, flowers and siliques. No GUS expression was detected in the stamens and seeds in *AtRPD3B:GUS* plants (Figure 4B). Quantitative GUS activity assay was also conducted in this study. GUS activity was observed from respective organs of the *AtRPD3A::GUS* and *AtRPD3B::GUS* transgenic plants (Figure 4 C, D). These results reveal potential importance of *AtRPD3A* and *AtRPD3B* in plants development.

2. The effects of hormones and wounding on the expression of AtRPD3A:GUS and AtRPD3B:GUS

The *AtRPD3A:GUS* and *AtRPD3B:GUS* transgenic plants were treated with 100 μM jasmonate (JA), 100 μM gibberellin (GA) or 100 μM ACC (an ethylene precursor) for 12 hours, as well as wounding (incision to the leaf). Both *AtRPD3A:GUS* and *AtRPD3B:GUS* were induced by JA, ACC and wounding (Figure 5). The wounding induction was a localized response, which was evident by the *GUS* staining around the margins of the incision. High levels of *GUS* expression could also be observed in *AtRPD3B:GUS* transgenic leaves after the GA treatment but not in the *AtRPD3A:GUS* leaves (Figure 5). JA and ACC are known participants and mediators of pathogen and stress response in plants (Ellis and Turner, 2001; Penninckx, 1998) and GA is known to be involved in essential developmental processes such as flowering and growth (Wilson et al, 1992; Putterill, et al, 1995; Blazquez et al, 1998). The induction of *AtRPD3A* and *AtRPD3B* by these hormones indicates important functions for *AtRPD3A* and *AtRPD3B* in *Arabidopsis*.

3. Deletion analysis of AtRPD3A and AtRPD3B promoters

To examine the essential regulatory regions in the *AtRPD3A* and *AtRPD3B* promoters, we generated several 5'-deletion constructs. For the *AtRPD3A* promoter, the 1378 bp promoter fragment was deleted from its 5' end to generate 1064 bp, 742 bp, 508 bp and 222 bp fragments, respectively (Figure 6A). These four truncated fragments were subcloned into the pCAMBIA1381 vector upstream to the *GUS* reporter and stably transformed into *Arabidopsis* using *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). For the *AtRPD3B* promoter, the 1357 bp sequence was deleted from its

5’ end to get 1017 bp, 757 bp and 374 bp fragments, respectively (Figure 6B). These three fragments were also subcloned into the pCAMBIA1381 vector upstream to the *GUS* reporter and transformed into *Arabidopsis*. Transgenic plants were selected on hygromycin selection medium and the mature rosette leaves from transgenic plants were used for the histochemical GUS assay.

No GUS activity was observed in the -*1064RPD3A:GUS*, -*742RPD3A:GUS*, -*508RPD3A:GUS*, and -*222RPD3A:GUS* transgenic plants (data not shown). This result indicates that the fragment from -1378bp to -1064bp is essential for the *AtRPD3A* promoter activity. For the *AtRPD3B* promoter, GUS activity was detected in the -*1017RPD3B:GUS* and -*757RPD3B:GUS* transgenic plants. GUS expression was completely abolished in the -*374RPD3B:GUS* (Figure 6C). This result indicates that the essential component for *AtRPD3B* promoter activity is in the region between -757bp to -374 bp.

4. AtRPD3A and *AtRPD3B promoter contains hormone and wounding responsive motifs*

We further analyzed both the *AtRPD3A* and *AtRPD3B* promoters by submitting the 1500 bp sequences upstream of the ATG translational start codon to the plantCARE database (http://oberon.fvms.ugent.be:8080/PlantCARE) for identify of putative *cis*-regulatory elements. Sequence analysis of the *AtRPD3A* and *AtRPD3B* promoter revealed that they contain a variety of motifs that may be involved in different hormone and stress response pathways. As shown in Table 3, the *AtRPD3A* promoter contained the CGTCA and TGACG motif, which in certain context, have been shown to be responsive to JA in

plants (Ouwerkerk and Memelink 1999; Park et al, 1999; Pasquali et al, 1999). Also, well characterized ethylene responsive elements (EREs) (Ohme and Shinshi, 1995; Roman et al, 1995; Kieber, 1997), P-box (gibberellin responsive element) (Jacobson and Close, 1991), ABRE (abscisic acid responsive element) (Finklestein and Rock**,** 2002) and WUN (wounding responsive element) (Baron and Zambryski, 1995) were among the *cis*-elements identified in the *AtRPD3A* promoter (Table 3). Similarly, the *AtRPD3B* promoter contained the ERE, P-box, ABRE and WUN motifs, but not the JA responsive elements (Table 4). The composition of *AtRPD3B* promoter is different from the *AtRPD3A* promoter, which is consistent with their different behavior in the spatial expression profile.

5. AtRPD3A and AtRPD3B are Localized in Nuclei

To investigate the cellular distribution of the AtRPD3A and AtRPD3B proteins, we performed in vivo targeting experiments using green fluorescent protein (GFP). *AtRPD3A-GFP* and *AtRPD3B-GFP* gene fusions driven by 35S promoter to achieve high levels of constitutive expression were created and introduced into *Arabidopsis* using *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). To confirm that the fusion proteins entered the nucleus, we monitored the fluorescence of GFP at the cellular level. Protoplasts were isolated from seedlings of transgenic *Arabidopsis*, and localization of the fusion proteins was determined under a fluorescence microscope. As shown in Figure 7, bright green fluorescence was observed throughout the nuclei in both transgenic lines. This indicates that AtRPD3A-GFP and AtRPD3B-GFP fusion proteins were localized in the nucleus of the *Arabidopsis* cells. This result supports the idea that these two proteins may be involved in transcription regulation.

Role of AtRPD3B in Leaf Senescence and JA Response

1. Analysis of axe1-5 mutant and AtRPD3B- RNAi plants

In order to identify the function of the *AtRPD3B*, we analyzed the *AtRPD3B* mutant, *axe1-5*, and two *AtRPD3B* interference (RNAi) lines, CS24038 and CS24039. The *axe1-5* mutant line is Columbia wild type back ground and carries a G to A point mutation in *AtRPD3B* 1635 bp downstream of the ATG translational start codon at the third exon-intron junction and it is derived from (Murfett et al, 2001; Probst et al, 2004) (Figure 8A). CS24038 and CS24039 were generated by expressing a fragment of the target gene *AtRPD3B* in an inverted repeat orientation for RNAi silencing (Plant Chromatin Database: http://chromdb.org). These two RNAi lines are derived from Wassilewskija (Ws) ecotype. RT-PCR was conducted to examine *AtRPD3B* transcript levels in mutant and wild type plants. Total RNA was isolated from Columbia wild type, *axe1-5*, Ws wild type, CS24038 and CS24039 plants. As shown in Figure 8B, *axe1-5*, CS24038 and CS24039 lines had lower or no *AtRPD3B* transcript accumulation as compared with their corresponding wild type plants. Western blot analysis with the acetylated histone H3 antibody was carried out to check for acetylation status in the wild type and mutant lines. As shown in Figure 8C, *axe1-5* and the two *AtRPD3B-RNAi* lines (CS20438/CS20439) had higher levels of H3 acetylation when compared with their wild type counterparts, suggesting that AtRPD3B transcript level affects histone acetylation levels globally. The weaker acetylation phenotypes of AtRPD3B mutants might result from redundancy of histone deacetylation function in the *Arabidopsis* genome.

2. AtRPD3B mutant leaves show increased leaf longevity

Initial phenotypic observation of *axe1-5* and two *AtRPD3B-RNAi* lines (CS2048/CS2043) revealed that they had delayed leaf senescence when compared to their wild type counterpart. To further analyze the role of *AtRPD3B* in leaf senescence, we examined leaf longevity of these mutants visually (Figure 9A). The phenotype of individual leaves was followed from the formation of a visually recognizable leaf primordial (1 mm in sized) (days after leaf emergence, DAE). The leaf was considered dead when the entire leaf turned yellow (Grbi and Bleecker, 1995). As shown in Figure 9A, the leaves of these mutants turned yellow much more slowly and showed increased leaf longevity when compared with their wild type counterparts.

Leaf longevity of the mutant was also assessed by measuring typical senescence-associated physiological markers, such as chlorophyll concentration and photochemical efficiency of photosystem II (PSII) (Fan et al, 1997; Oh et al, 1997). Chlorophyll content is the first to decline at the onset of senescence (Nam, 1997) and it is considered as an important indicator of the rate of senescence (Kleber-Janke and Krupinska, 1997). Chlorophyll content of the 6th rosette leaf was measured from 15 DAE (when the 6th rosette leaves were fully grown). The chlorophyll content was tracked at an interval of every 10 days. At 45 DAE, the leaves of Ws and Columbia wild type lost 65-75% of their chlorophyll, whereas *axe1-5* and the two AtRPD3B-RNAi lines just lost 15-35% (Figure 9B). Delayed senescence of the mutants was also measured by the delay in the decrease in photosynthetic activity (Figure 9C). Photosystem II efficiency has been shown to be an effective indicator of leaf senescence in plants (Lu and Zhang, 1998). It was demonstrated that during senescence, the PSII efficiency declines rapidly leading to a

loss of photosynthetic capabilities of the leaves and eventual death of the leaves. *axe1-5*, CS24038 and CS24039 leaves consistently showed later development of senescence-associated changes. These results suggest that decreased expression of AtRPD3B causes increased leaf longevity in *Arabidopsis*.

Leaf senescence is accompanied by decreased expression of genes related to photosynthesis and protein synthesis genes (PAGs) (Bate et al, 1991) and increased expression of senescence-associated genes (SAGs) (Nam, 1997). To determine the effect of *axe1-5*, CS24038 and CS24039 on gene expression, we examined the expression patterns of these genes during leaf development (Figure 10). Specifically, *SAG12* has been shown to be upregulated in an age-dependant manner and is minimally regulated by environmental factors (Gan and Amasino, 1997). Another important SAG, which is upregulated during senescence, is senescence associated protein, *SEN4* (Park *et al*, 1998). One of the important PAGs is the plastid ribosomal protein small subunit 17 (RPS17) (Woo et al, 2002). RT-PCR results revealed that *SAG12* and *SEN4* were downregulated in *axe1-5*, CS24038 and CS24039 when compared with their corresponding wild types. In comparison, chloroplast ribosomal protein S17 (*RPS17*) was upregulated in the *AtRPD3B* mutant lines (Figure 10). These results support the idea that AtRPD3B may be required for SAGs expression and therefore it is involved in senescence progression. In the absence of *AtRPD3B*, PAG genes are upregulated, which leads to a higher rate of photosynthesis, resulting in higher PSII efficiency and higher chlorophyll content in the *AtRPD3B* mutants.

3. AtRPD3B mutants are hyper-sensitive to JA

Leaf senescence is regarded as a developmentally programmed event that can be modulated by a range of plant hormones, such as ABA, JA, and ethylene (Weidhase et al, 1987; Zeevaart and Creelman, 1988; Aharoni, 1989). Apart from endogenous aging signals, JA has been implicated in playing a major role in enhancing senescence (He et al, 2002) because an intact JA pathway is required for normal senescence progression. Additionally, it was demonstrated that COI1, a JA response protein and an F-box protein, may recognize *AtRPD3B* as a target, suggesting the possible role of *AtRPD3B* in JA signaling (Devoto et al, 2002).

To determine whether AtRPD3B is involved in JA response, a JA sensitivity assay was conducted. Columbia wild type, *axe1-5*, Ws wild type, *CS20438* and *CS20439* seeds were germinated on MS media supplemented with various concentrations of JA. As shown in Figure 11B, *axe1-5*, *CS20438* and *CS20439* seed germination rate decreased dramatically at 75 μM JA. In comparison, the germination of Columbia and Ws wild type was not affected at this concentration of JA. Root elongation of the *axe1-5*, *CS20438* and *CS20439* lines dropped by 35-55% at 10 μM of JA, whereas Columbia and Ws wild type root elongation decreased by only 10-25% (Figure 11A and Figure 11C). As shown in Figure 11D, fresh weights of the *AtRPD3B* mutants treated with JA declined more rapidly with increasing concentrations of JA when compared with their wild type counterparts. At 10 μM of JA, the fresh weights of *axe1-5*, *CS24038* and *CS20439* seedlings were reduced by 50-60%, whereas that of the wild types was reduced by 30%. These results indicate that *AtRPD3B* mutants are hyper-sensitive to JA.

4. The expression of JA response genes in the axe1-5 and AtRPD3B-RNAi plants

To investigate whether *AtRPD3B* expression is affected by JA in Columbia wild type and the *axe1-5* mutant, plants were treated with JA and RT-PCR was conducted. In wild type plants there was an increase in *AtRPD3B* transcript level upon JA treatment, which is similar to the JA responsive genes, *PDF1.2* (pathogen defense gene), *VSP2* (vegetative storage protein), *JIN* (JA insensitive) and *ERF1* (ethylene response factor). However, in *axe1-5* mutant plants, the expression of these JA responsive genes was unchanged (Figure 12).

To further investigate the role of AtRPD3B in JA responsive pathway, RT-PCR was conducted to examine transcript levels of JA responsive genes, *PDF1.2*, *JIN1*, *VSP2* and *ERF1*. We observed that the JA response genes were downregulated in *axe1-5*, CS24038 and CS24039. *PDF1.2* is a downstream gene of the JA response pathway and is involved in defense to pathogen attack. PDF1.2 (Xu et al, 1994; Penninckx et al, 1996; Penninckx et al, 1998) is constitutively upregulated in JA hyper-biosynthesis mutant *cev1* (constitutive overexpression of *VSP*) (Ellis and Turner, 2001). *VSP2* is also upregulated in the *cev1* mutant. Additionally, *JIN1* is another important downstream gene in the JA signal pathway (Berger and Mullet, 1996). ERF1 represents a transcription factor that regulates pathogen response genes and is also an important point of convergence between JA and ET response pathways (Lorenzo et al, 2003). All these four genes were down-regulated in *axe1-5*, CS24038 and CS24039 mutant lines (Figure 13) indicating that *AtRPD3B* might be required for their expression.

JA is known to be involved in several developmental processes such as pollen formation, anther dehiscence and fruit ripening (Devoto and Turner, 2003). The *coi1-1* mutant is male sterile and lacks the expression of JA induced genes, including *VSP2* and the plant defense related genes, *Thi2.1* and *PDF1.2* (Feys et al., 1994; Benedetti et al, 1995; Penninckx et al, 1998; Xie et al, 1998). As shown in Figure 14, siliques of *axe1-5*, CS24038 and CS24039 are smaller and have fewer seeds when compared with those of their wild type counterparts. We also observed that the anther was unable to dehisce and there was no pollens on the stigma of the mutant plants. These phenotypes revealed partial sterility of *axe1-5* and AtRPD3B-RNAi plants (Figure 14). This further supports that AtRPD3B may have a role in JA regulatory processes.

Role of AtRPD3B in Flowering Pathway

1. axe1-5 and AtRPD3B-RNAi plants have delayed flowering

To assess of the role of AtRPD3B in flowering, we analyzed the flowering time of the *axe1-5* and AtRPD3B-RNAi plants. The *axe1-5* and AtRPD3B-RNAi plants showed later flowering phenotypes, as measured by the days when the first flower bud was visible (Table 5, Figure 15). Additionally, delayed flowering leads to excessive vegetative proliferation, which leads to an increase in the rosette leaf numbers (Nooden and Penny, 2001). Therefore, we also recorded the number of rosette leaves of *axe1-5*, CS24038 and CS24039 as compared with Columbia and Ws wild types (Table 5). Plants were grown under different photoperiod conditions: long-day (LD, 16 hours light/8 hours dark) and short day (SD, 8 hours light/16 hours dark). The flowering was greatly delayed in short-day as well as in long-day in terms of both the days to flowering and the rosette

leaf numbers at flowering initiation. *axe1-5* did not flower even at 140 day after germination in short-day, when some rosette leaves showed senescence. These observations suggest that AtRPD3B is involved in flowering.

2. AtRPD3B is involved in the autonomous flowering pathway

The flowering time and rosette leaf numbers of *axe1-5* and AtRPD3B-RNAi plants suggested that they delayed flowering in long-day as well as in short-day conditions. Because the flowering mutants of photoperiod or long day pathway have mutant phenotype only under long-day conditions, but behave like wild type under short-day conditions (Mouradov and Coupland, 2002), the *AtRPD3B* mutants are therefore not photoperiod mutants.

The involvement of *AtRPD3B* in the vernalization pathway was investigated by growing *axe1-5*, CS24038 and CS24039 after 42 days vernalization. Without vernalization, the *axe1-5*, CS24038 and CS24039 lines flowered much later when compared with their corresponding wild types, indicated by days to flowering and rosette leaf number (Figure 16 and Figure 17A, B). *axe1-5*, CS24038 and CS24039 plants started to flower when they had 16, 8 and 9 leaves, respectively. In comparison, Columbia and Ws wild type had 9 and 6 leaves at flowering. After vernalization, the *axe1-5*, CS24038 and CS24039 plants flowered at almost the same time as their wild type counterparts, indicating that delayed flowering phenotype can be rescued by vernalization (Figure 16 and Figure 17A, B). Similar results were obtained under short-day (Figure 17C). The autonomous pathway mutants can be rescued by vernalization (Mouradov and Coupland, 2002), whereas the vernalization pathway mutants cannot be rescued by vernalization.

Therefore, the AtRPD3B is not involved in vernalization pathway. When we tested whether *AtRPD3B:GUS* expression can be affected by a 42-day vernalization treatment, no difference was found in GUS activity after vernalization (data not shown).

3. The expression of FLC and SOC1 are affected in axe1-5 and AtRPD3B-RNAi plants

The delayed flowering of *axe1-5*, *CS24038* and CS24039 plants promoted us to analyze whether the expression of *FLC* (*FLOWERING LOCUS C*) was affected. The MADS domain–containing transcription factor *FLC* acts as an inhibitor of flowering and is a convergence point for several pathways that regulate flowering time in Arabidopsis (Koornneef et al, 1994; Lee et al, 1994; Sanda and Amasino 1996b; Michaels and Amasino 1999 and Sheldon et al, 1999). RT-PCR was conducted to examine the expression level of *FLC* as well as other flowering related genes, *SOC1* and *FT*. SOC1 is a MADS domain–containing transcription factor that acts as a promoter of flowering (Borner et al, 2000; Lee et al, 2000; Samach et al, 2000) and *FLC* acts as a negative regulator of *SOC1* (Michaels and Amasino, 2001). In the *AtRPD3B* mutants, *FLC* was upregulated, whereas *SOC1* was markedly downregulated (Figure 18). Another flowering promoting gene, *FT*, was not affected, indicating that *AtRPD3B* might affect flowering time by specifically targeting *SOC1* via *FLC* repression (Figure 18).

Microarray Analysis of gene expression in *35S:AtRPD3A* Plants

To identify genes regulated by *AtRPD3A*, we conducted a microarray analysis using RNA samples from *AtRPD3A* overexpression lines (*35S: AtRPD3A*) and wild type plants. 362 genes were found that had >1.5 or <0.66 fold change and P-value ≤0.05. These 362 genes were classified into 9 functional categories (Figure 19). 18.5% of

affected genes belonged to the stress and defense response category (Table 6) and 25% of genes belonged to energy and metabolism category. The identification of stress response genes as major targets of *AtRPD3A* suggest the possibility of the involvement of *AtRPD3A* in stress response pathways such as those mediate by hormones such as JA, ethylene and ABA. Our recent study revealed that the expression of *HDA19* (*AtRPD3A*) was induced by wounding, the pathogen *Alternaria brassicicola*, and the plant hormones JA and ethylene (Zhou et al, 2005). Nevertheless a large percentage of affected genes in the *AtRPD3A* overexpression line are 'unclassified' and their specific functions remained to be identified. This would provide further insight as to the function of *AtRPD3A*. Additionally, genes involved in transcription also constituted significant proportion 13.8% of affected genes.

Table 3. *cis*-elements in *AtRPD3A* promoter

Site Name	Position	Sequence	Function
ABRE	-1189, -1187	gggACGTgga, gACGTg	abscisic acid responsiveness (Yamaguchi et al, 1994; Baker et al, 1994).
ERE	-682, -944	ATTTcaat, ATTTtaaa	ethylene-responsive element (Itzhaki et al, 1994)
P-box	-1345, -376, -748, -438, -996	CCTTtgg, CCTTttc, CCTTttc, CCTTtag	gibberellin-responsive element (Kim et al, 1992; Washida et al, 1994)
TATC-box	-261	TATCcaa	gibberellin-responsiveness (lu et al, 2002)
TCA-element	-294, -288, -122	AAGAAgaaga, cAGAAaaaga gAGAAgagta	salicylic acid responsiveness (Pastuglia et al, 1997).
TGACG-motif	-1335, -950, -563	TGACg	JA-responsiveness (Park et al, 1999; Pasquali et al, 1999)
WUN-motif	-1322, -721, -684, -618, -513	CATT	wound-responsive element (Pastuglia et al, 1997).

Table 4. *cis*-elements in *AtRPD3B* promoter

Site Name	Position	Sequence	Function
ABRE	-822, -820	aggACGTggt, gACGTg	abscisic acid responsiveness (Yamaguchi et al, 1994; Baker et al, 1994).
Box-W1	-1015, -793	TTGAcc	fungal elicitor responsive element (Rushton et al, 1996)
ERE	-582	ATTTctaa	ethylene-responsive element (Itzhaki et al, 1994).
P-box	-1095, -566	CCTTtag, CCTTttt	gibberellin-responsive element (Kim et al, 1992; Washida et al, 1994).
TATC-box	-1349	TATCcct	gibberellin-responsiveness (Jacobson and Close, 1991)
TCA-element	-1336, -1271, -942, -607, -604, -601, -598	aAGAAgaagc, aAGAAgaaga	salicylic acid responsiveness (Pastuglia et al, 1997).
WUN-motif	-1358, -1278, -1258, -1157, -946, -617, -484, -503, -346, -232, -198, -154, -107	AATT, GATT, CATT, TATT	wound-responsive element (Pastuglia et al, 1997).

*Numbers represent the nucleotide position relative to the translation starting site (+1).

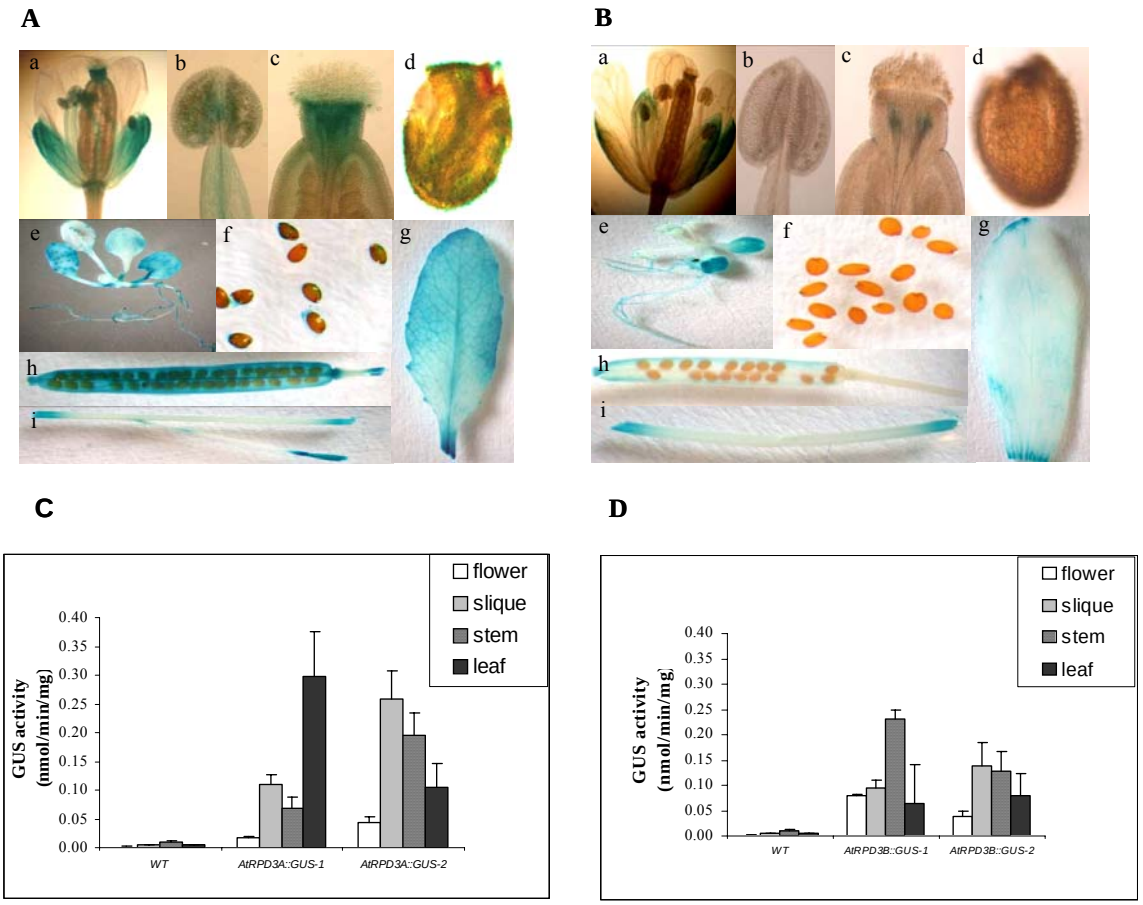


Figure 4. Comparison of the *AtRPD3A:GUS* and *AtRPD3B:GUS* expression.

A and B. Expression pattern of *AtRPD3A:GUS* (A) and *AtRPD3B:GUS* (B) in flower (a), anther (b), stigma (c), seeds (d and f), silique (h), mature leaves (g), stems (i) and 2-weed-old seedling (e).

C and D. Quantitative GUS activity of *AtRPD3A:GUS* (C) and *AtRPD3B:GUS* (D). Total protein was extracted form different organs and GUS activity was assayed. As a control Columbia wild type (WT) plants were also analyzed.

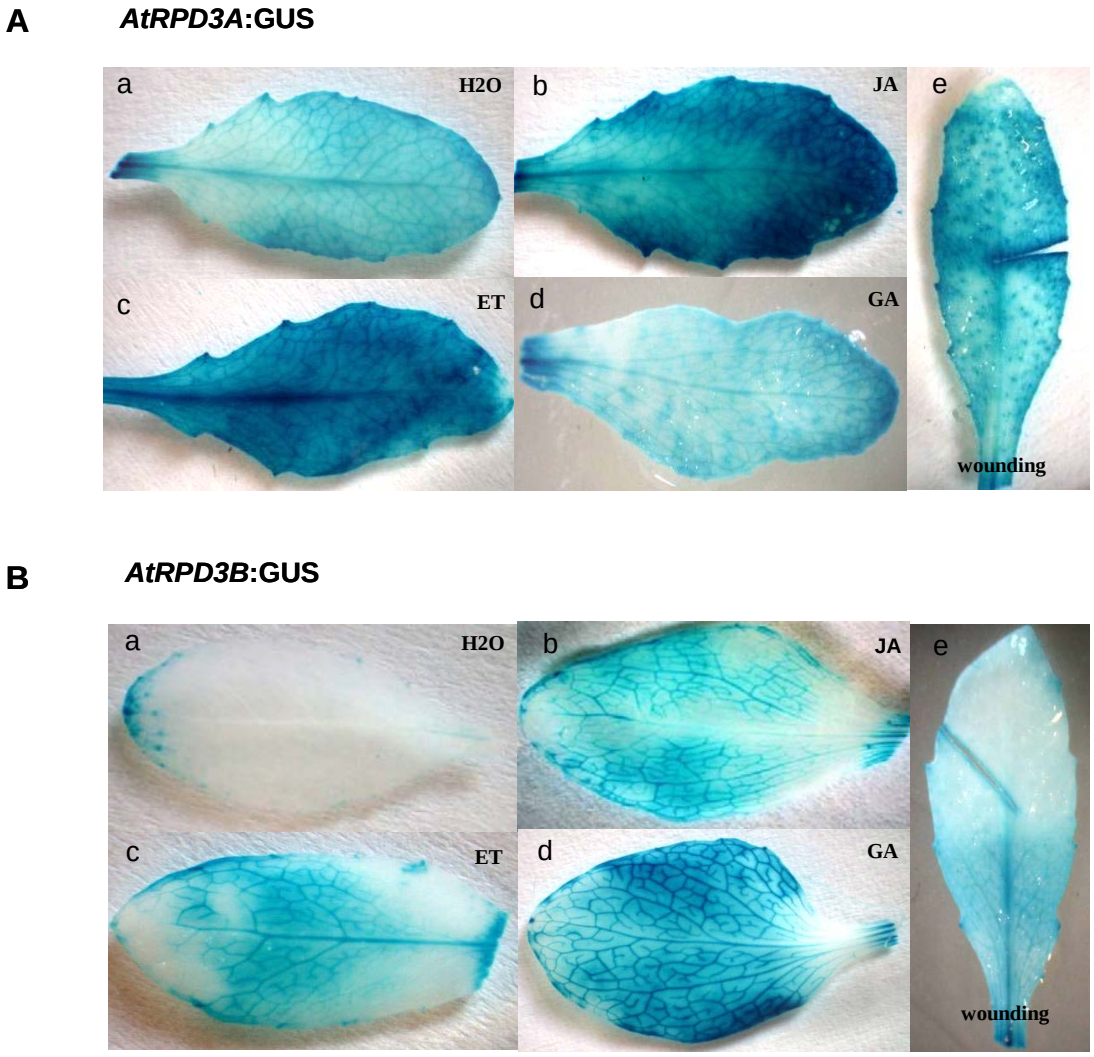


Figure 5. Induction of *AtRPD3A:GUS* and *AtRPD3B:GUS* by plant hormones and wounding.

GUS expression of *AtRPD3A:GUS* (A) and *AtRPD3B:GUS* (B) treated with H2O (a), 0.1mM jasmonic acid (JA) (b), 100 μM ethylene (ET) (c), 100 μM gibbrellic acid (GA) (d) and wounding (e).

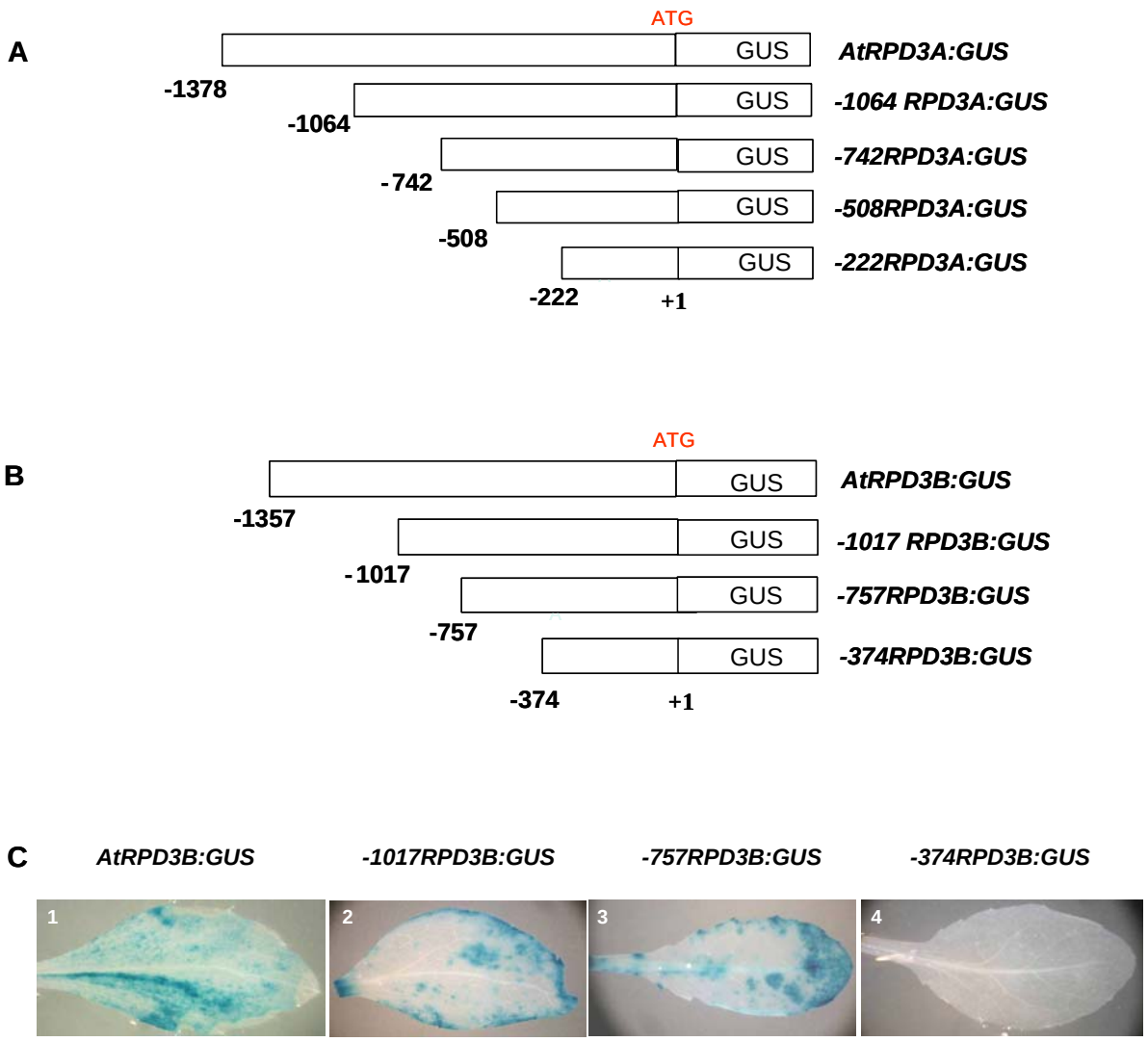


Figure 6. Deletion analysis of *AtRPD3A* and *AtRPD3B* promoters.

A and B. Schematic representation of sequential 5' end deletions created in the *AtRPD3A* (A) and *AtRPD3B* (B) promoter sequences. Numbers represent the nucleotide position relative to the translation starting point (+1).

C. Histochemical GUS activity assay of *AtRPD3B* promoter deletion transgenic plants. GUS activity can be observed in *AtRPD3B:GUS*, *-1017RPD3B:GUS* and *-757RPD3B:GUS*. GUS expression was completely abolished in *-374RPD3B:GUS*.

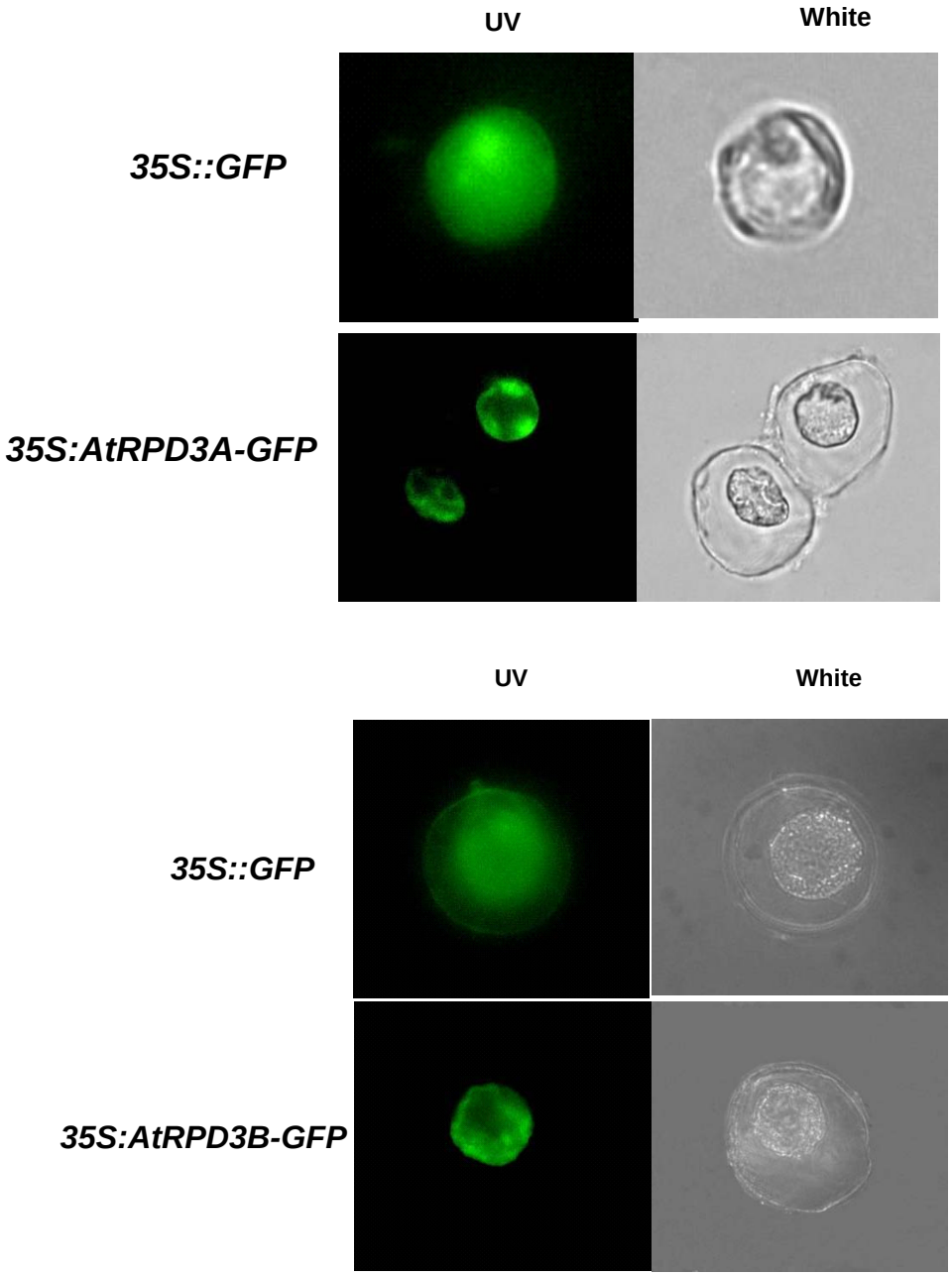


Figure 7. Subcellular localization of AtRPD3A and AtRPD3B protein.

Protoplasts were isolated from the leaves of *35S::GFP*, 35S*::AtRPD3A-GFP* and 35S*::AtRPD3B-GFP* transgenic *Arabidopsis* plants. GFP fluorescence was examined using a fluorescence microscope under UV light and white light.

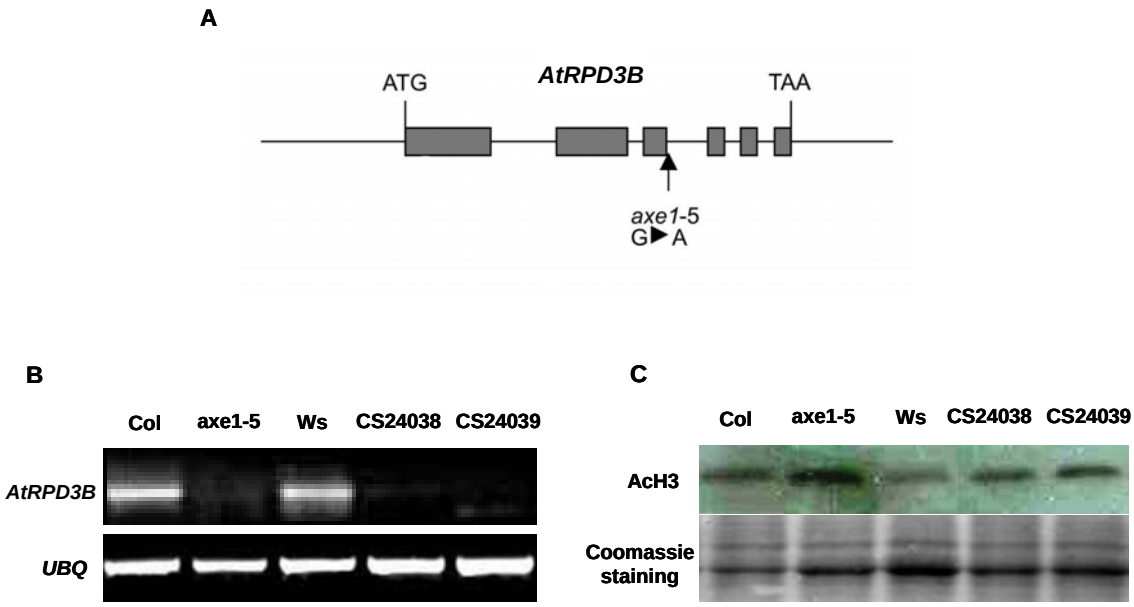


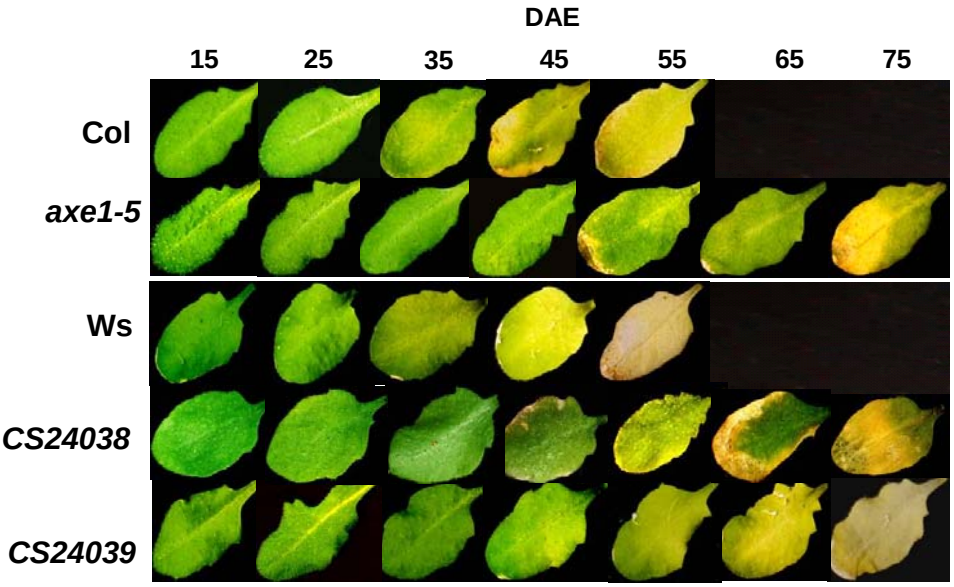
Figure 8. Expression of *AtRPD3B* and levels of tetra-acetylated H3 in *axe1-5* and *AtRPD3B-RNAi* plants.

A. Schematic representation of *AtRPD3B*. *axe1-5* has a base substitution of G to A at position 1635 downstream of the ATG at the third exon-intron junction. Exons are represented by black boxes.

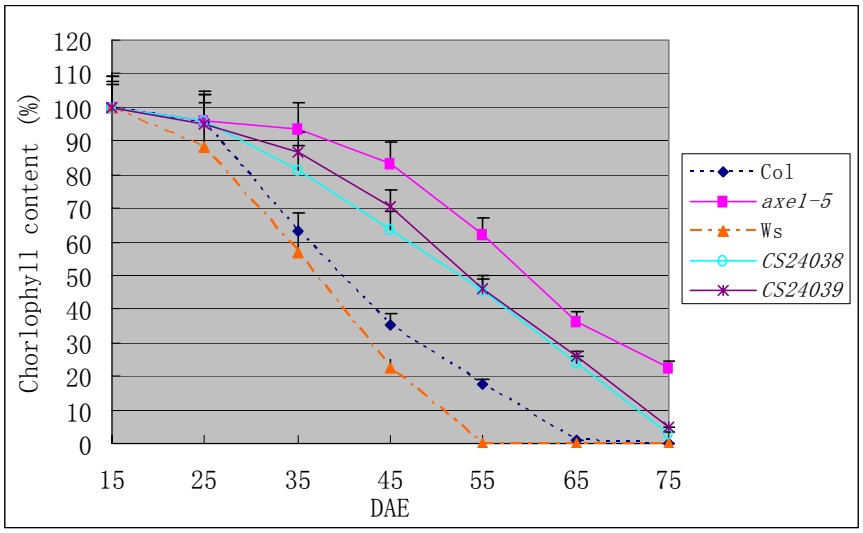
B. RT-PCR analysis to examine the expression of the *AtRPD3B*. Total RNA was isolated from leaf tissues of 3-week-old plants. *Ubiquitin* (*UBQ*) serves as internal control.

C. Western blot analysis to determine tetra-acetylated H3 (AcH3) (top panel) using AcH3 antibodies on protein extracts from different lines. Bottom panel is coomassie staining showing equal protein loading.

A


DAE
15
25
35
45
55
65
75
Col
axe1-5
Ws
CS24038
CS24039

B


Chorlophyll content (%)
120
110
100
90
80
70
60
50
40
30
20
10
0
15
25
35
45
55
65
75
DAE
Col
axe1-5
Ws
CS24038
CS24039

C

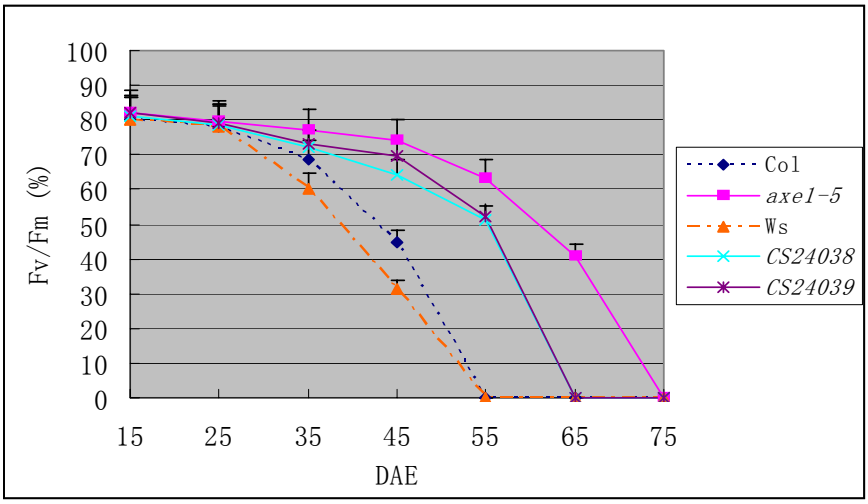

Fv/Fm (%)
100
90
80
70
60
50
40
30
20
10
0
15
25
35
45
55
65
75
DAE
Col
axe1-5
Ws
CS24038
CS24039

Figure 9. Age-dependent senescence phenotype in *axe1-5* and AtRPD3B-RANi (CS24038 and CS24039) plants.

A. The age-dependent senescence phenotype of Columbia wild type (Col), *axe1-5*, Ws wild type (Ws), CS24038 and CS24039 leaves. Photographs show representative leaves at each time point. Pictures were taken every 10 days from 15 DAE.

B. Chlorophyll content. The graph shows the percentage of chlorophyll content relative to 15 DAE.

C. Photochemical efficiency of PSII. *Fv/Fm values were measured every 10 days from 15 DAE, which is the day that the 6th rosette leaf is just fully grown. Error bars indicate SE ($n \geqslant 15$).

*Fv/Fm: maximum quantum yield of PSII electron transport (maximum variable fluorescence/maximum yield of fluorescence).

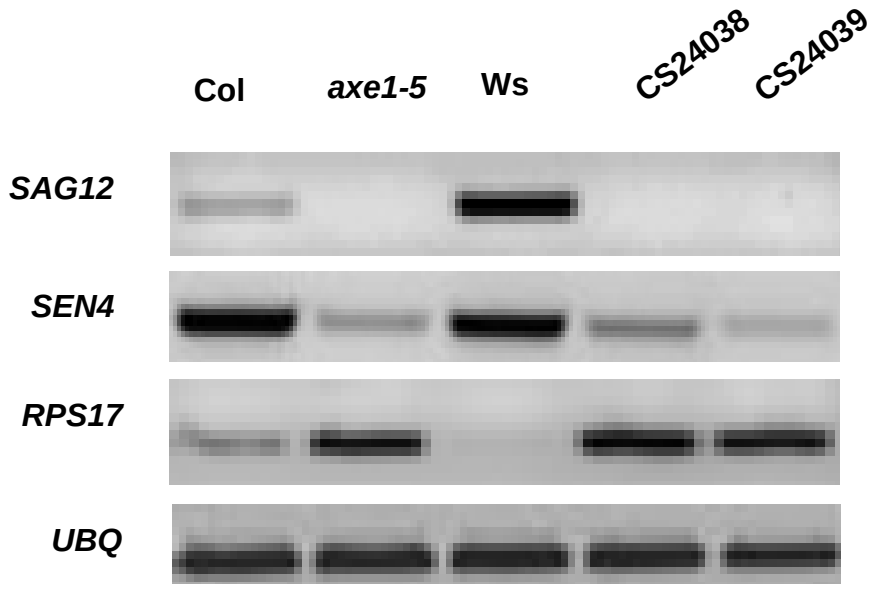


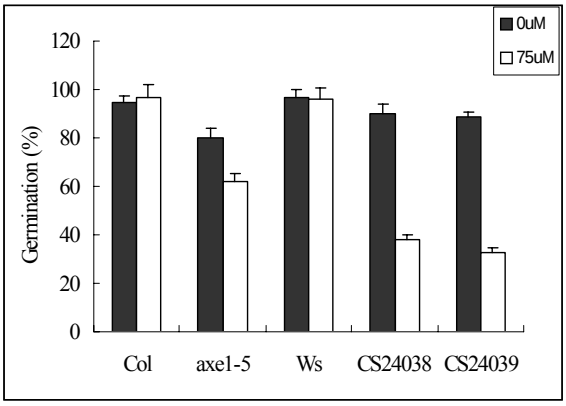
Figure 10. RT-PCR analysis of senescence marker genes.

SAG12, SEN4 and *RPS17* expression level were measured in Columbia wild type (Col), *axe1-5* mutant, Ws wild type (Ws) and two *AtRPD3B-RNAi* lines (CS24038 and CS24039). Total RNA for RT-PCR analysis was isolated from leaf tissues. *Ubiquitin* (*UBQ*) was shown as internal control.

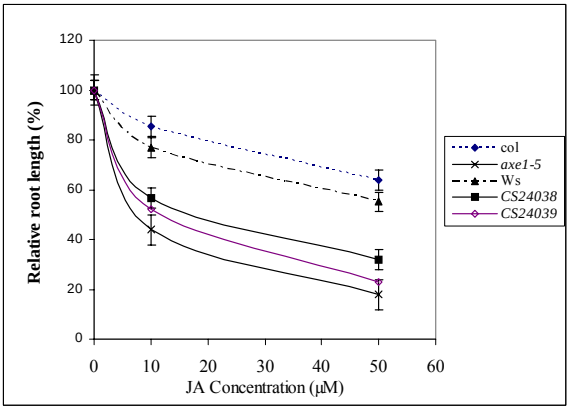
A


-JA
+JA
Col
axe1-5
Ws
CS24038
CS24038
Col
axe1-5
Ws
CS24038
CS24039

B


0uM
75uM
Germination (%)
120
100
80
60
40
20
0
Col
axe1-5
Ws
CS24038
CS24039

C


Relative root length (%)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
JA Concentration (μM)
col
axe1-5
Ws
CS24038
CS24039

D

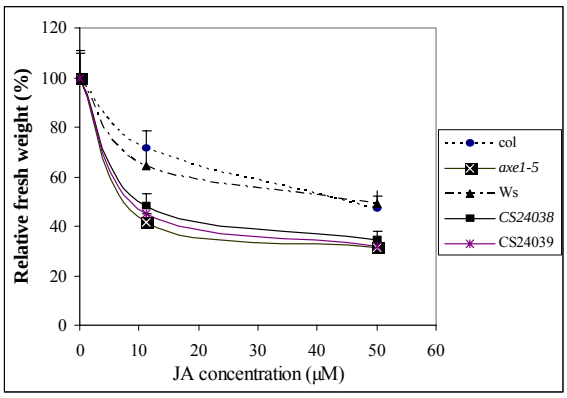

Relative fresh weight (%)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
JA concentration (μM)
col
axe1-5
Ws
CS24038
CS24039

Figure 11. *axe1-5* and two AtRPD3B-RNAi lines (CS24038 and CS24039) are hypersensitive to JA.

A. Phenotypes of 7-day-old Columbia wild type (Col), *axe1-5*, Ws wild type (Ws), CS24038 and CS24039 grown on MS media without (-JA) or with (+JA) 10 μM JA. Photographs show representative seedlings.

B. Seed germination rate comparison. Seeds were germinated on MS media without JA or with 75 μM JA. Germination rate was counted 7 days after germination.

C and D. JA dose-response curve of root length (C) and fresh weight (D). Root length or fresh weight of the seedlings grown on MS containing 1, 10 or 50 μM JA is expressed as a percentage of root length or fresh weight on MS (control). Error bars represent SE ($n \geqslant 20$).

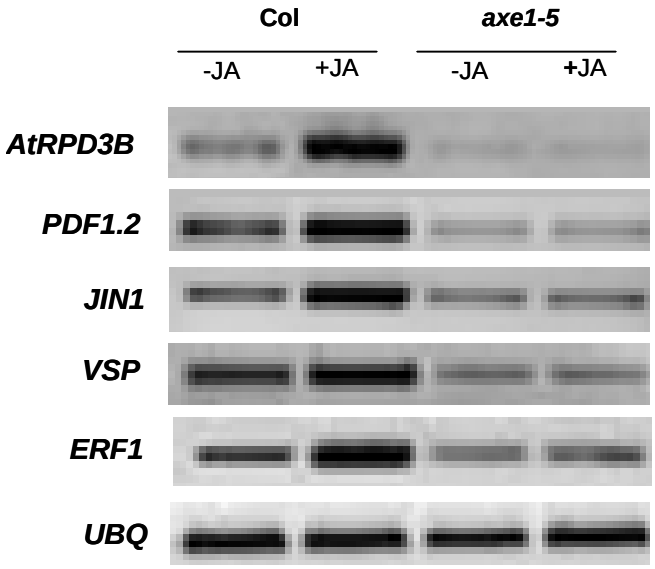


Figure 12. RT-PCR analysis of JA responsive genes.

RNA was isolated from 3-week-old Columbia wild type and *axe1-5* plants with (+JA) or without (-JA) 100 μM JA treatment. *Ubiquitin* (*UBQ*) was shown as internal control.

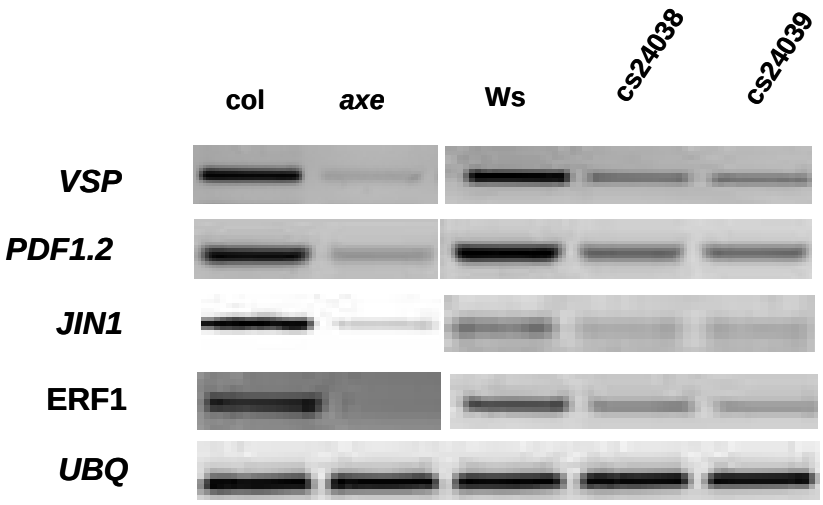


Figure 13. RT-PCR analysis of JA response genes.

RNA was isolated from 3-week-old plants of Columbia wild type, *axe1-5*, Ws wild type, CS24038 and CS24039. *VSP2*, *PDF1.2, JIN1* and *ERF1* had lower expression in all the mutant lines as compared with Columbia and Ws wild types. *Ubiquitin* (*UBQ*) serves as internal control.

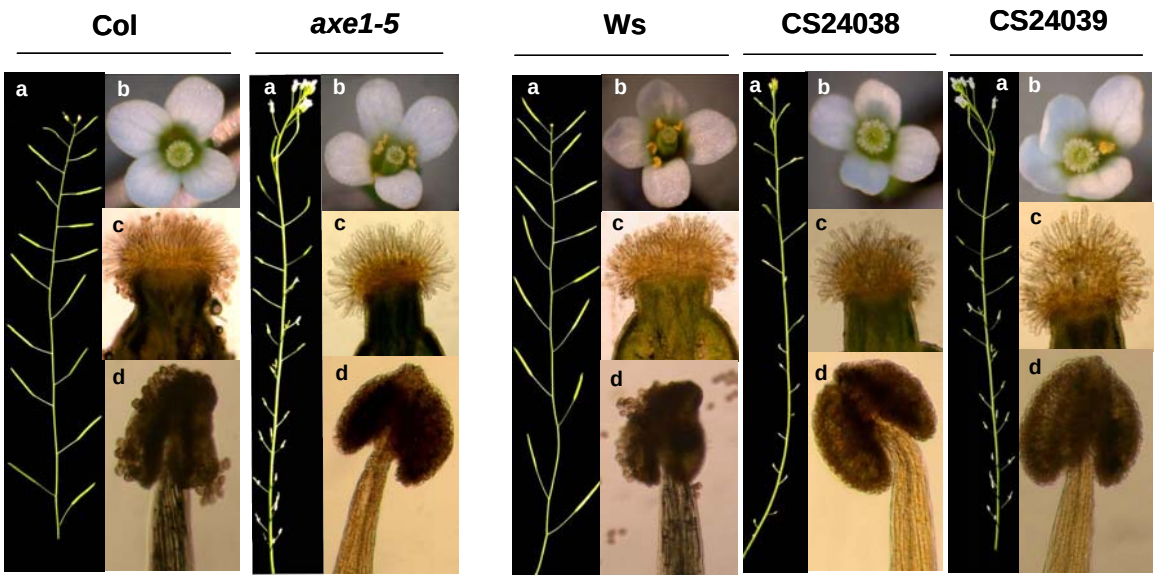


Figure 14. Flower phenotype of *axe1-5* and AtRPD3B-RNAi (CS24038 and CS24039) plants.

Photographs of mature siliques (a), fully opened flowers (b), stigma (c) and anther (d). Siliques of *axe1-5*, CS24038 and CS24039 are smaller and have fewer seeds when compared with their wild type counterparts. Anthers were unable to dehisce and no pollen was observed on the stigma of mutant plants, suggesting partial sterility of *axe1-5* and AtRPD3B-RNAi (CS24038 and CS24039) plants.

Table 5. Leaf number and flowering time (days) of *axe1-5* and *AtRPD3B-RNAi* (CS24038 and CS24039) plants.

Genotype	Rosette leaf number*			Flowering time (days)		
	*24 hours**	*long-day**	*short-day**	*24 hours*	*long-day*	*short-day*
Columbia	7.7±0.9	9.3±0.5	26.3±0.7	28.6±0.9	31.5±1.2	99.4±0.9
axe-5	11.3±1.3	15.2±1.0	75.2±3.2**	33.3±2.5	37.3±1.7	>140**
Ws	6±1.1	5.7±1.0	22±0.7	24.1±0.7	23.2±0.8	56.0±1.2
CS24038	7±0.9	8.1±0.7	50±1.7	26.3±1.0	27.3±1.0	118.4±3.9
CS24039	8.1±1.0	10.3±1.1	51.9±1.0	29.3±1.7	34.3±2.6	122.6±4.2

*Total leaf number ±SE when the first flower was observed after seed germination. Columbia wild type (Col), *axe1-5* Ws wild type (Ws), CS24038 and CS24039 plants were grown under 24 hours light, long-day (16 hours light/ 8hours dark) and short-day (8 hours light/ 16 hours dark) respectively. Values are the mean of 25 plants per phenotype.

** *axe1-5* plants did not flowering 140 days after germination under short-day. The rosette leaf number of *axe1-5* was counted at 140 days.

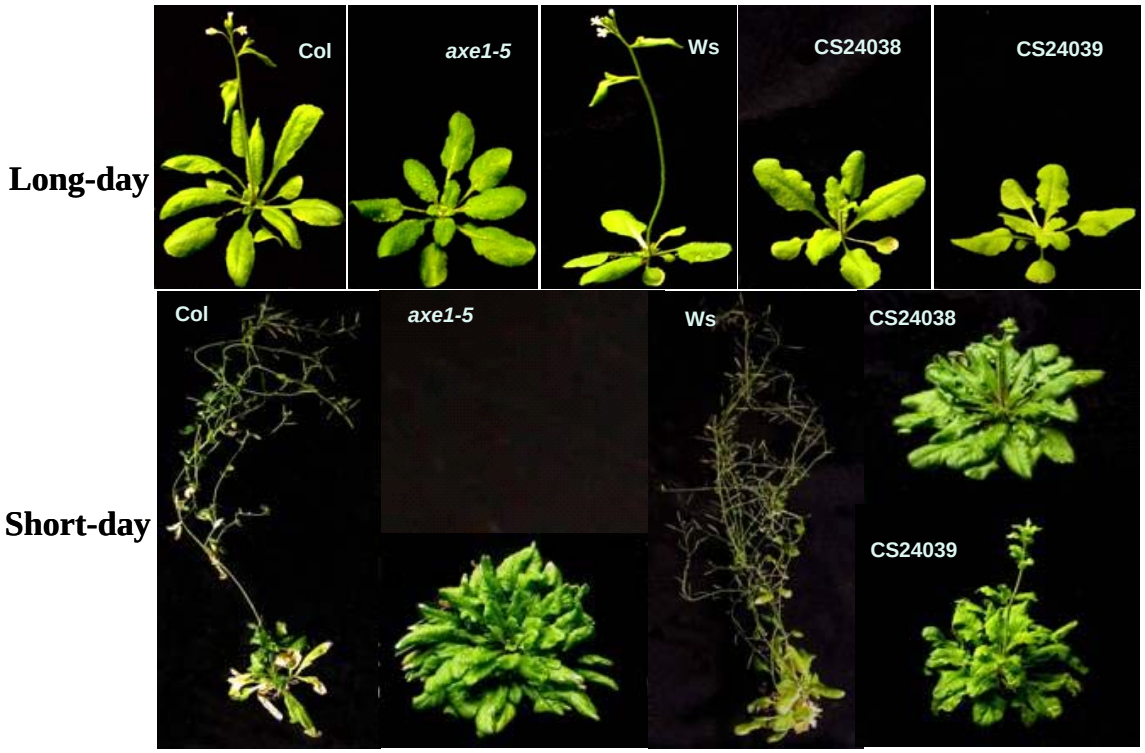


Figure 15. Flowering phenotypes of *axe1-5* and *AtRPD3B*-RNAi (CS24038 and CS24039) plants.

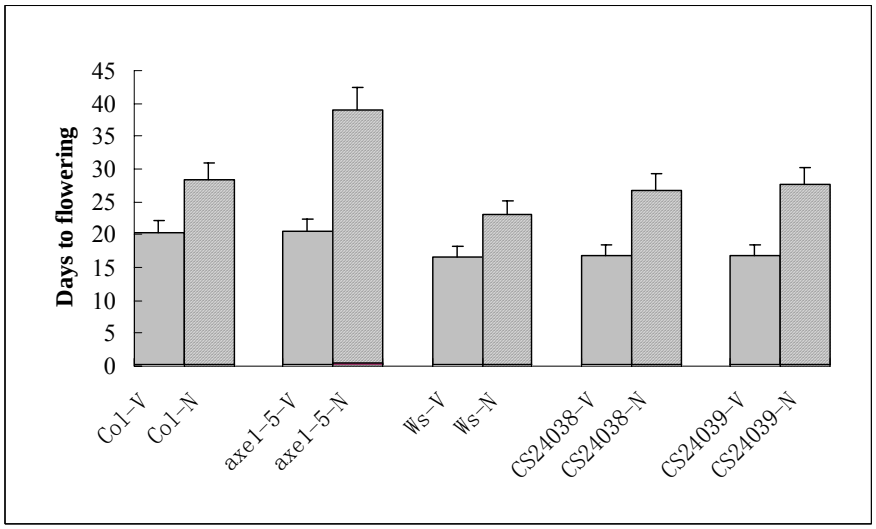
Plants were grown under long-day (16 hours light and 8 hours dark) or short-day (8 hours light and 16 hours dark) conditions. Photographs show representative plants for Columbia ecotype at 30 days and 23 days for Ws ecotype in long-day, and 120 days after seed germination in short-day.

Figure 16. Flowering phenotype of *axe1-5* and *AtRPD3B*-RNAi (CS24038 and CS24039) plants.

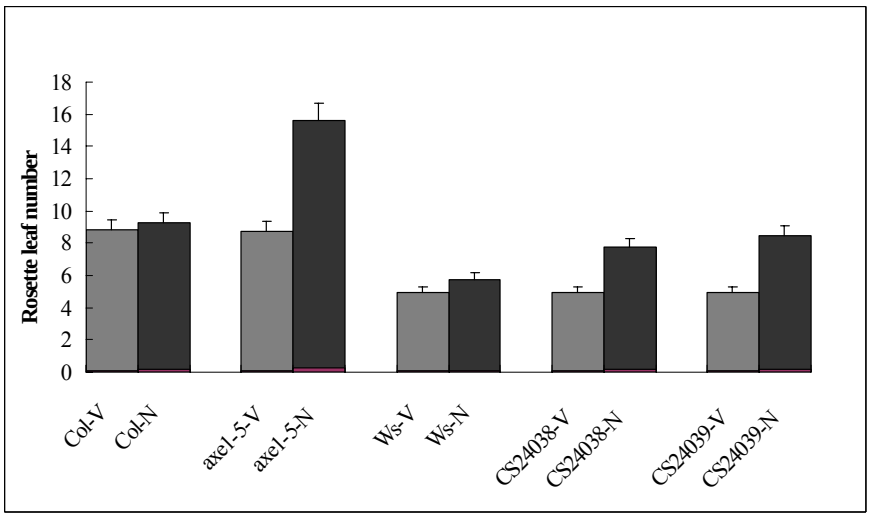
A. Plants were grown under long-day condition without vernalization. Photographs show representative plants at 30 days for Columbia ecotype and 23 days for Ws ecotype.

B. Plants were grown under long-day condition with 42-day vernalization treatment. Photographs were taken at 21 days for Columbia ecotype and 18 days for Ws ecotype after the plants were moved from -4ºC to 22ºC long-day condition.

A


Days to flowering
45
40
35
30
25
20
15
10
5
0
Col–V
Col–N
axe1–5–V
axe1–5–N
Ws–V
Ws–N
CS24038–V
CS24038–N
CS24039–V
CS24039–N

B


Rosette leaf number
18
16
14
12
10
8
6
4
2
0
Col-V
Col-N
axe1-5-V
axe1-5-N
Ws-V
Ws-N
CS24038-V
CS24038-N
CS24039-V
CS24039-N

C

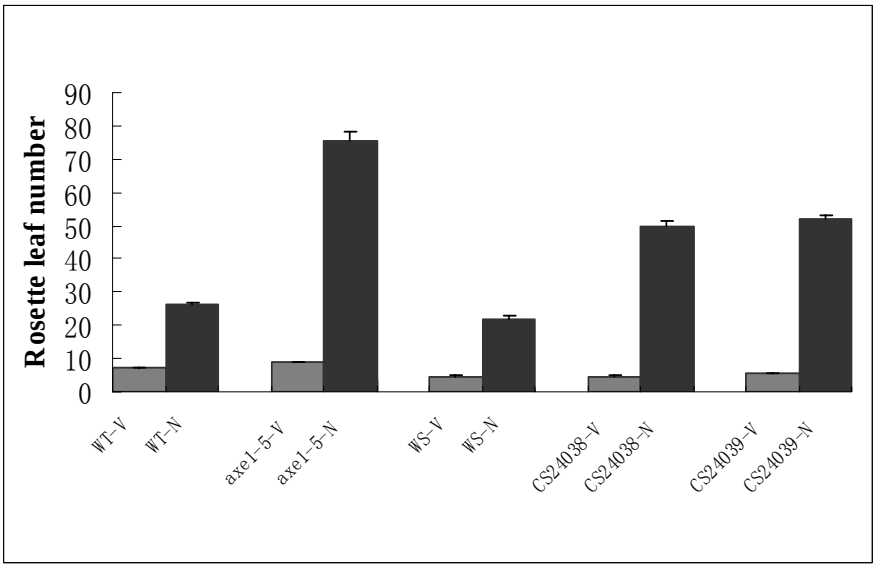

Rosette leaf number
90
80
70
60
50
40
30
20
10
0
WT–V
WT–N
axe1–5–V
axe1–5–N
WS–V
WS–N
CS24038–V
CS24038–N
CS24039–V
CS24039–N

Figure 17. Flowering time of wild type and *AtRPD3B*-RNAi lines (CS24038 and CS24039).

A. Days to flowering under long-day condition. Day to flowering data were recorded when the first flower bud was observed on plant.

B. Rosette leaf numbers under long-day.

C. Rosette leaf numbers under short-day.

Plants were grown under long-day (16 hours light/8 hours dark) (A and B) condition or short-day (8 hours light/16 hours dark) (C) condition with 42 days vernalization treatment **(V)** or without vernalization treatment **(N)**.

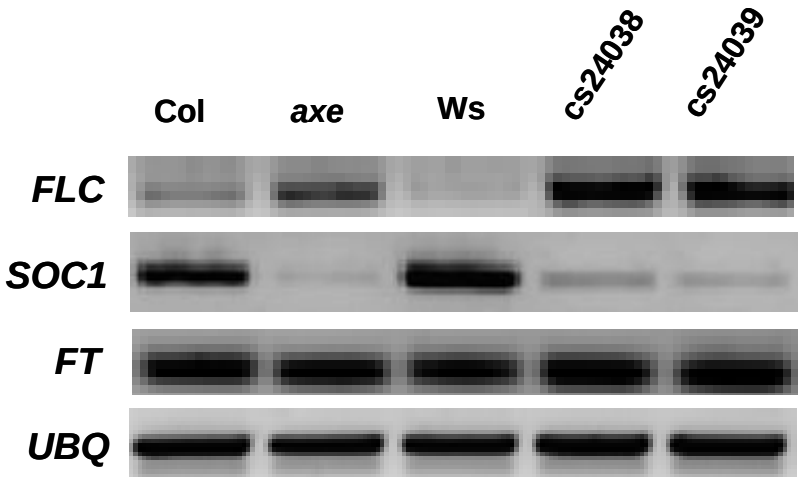


Figure 18. RT-PCR analysis of flowering pathway genes.

RNA was isolated from 3-week-old Columbia wild type, *axe1-5*, Ws wild type, CS24038 and CS24039 plants. *Ubiquitin* (*UBQ*) was shown as internal control. *FLC* was upregulated and *SOC1* was downregulated in mutant lines. These was no difference in *FT* expression among all the lines.

A

Classifications	# of genes	Percentage
Energy and metabolism	90	24.9
***Stress and defense**	66	18.2
Protein synthesis and destination	20	5.5
Signal transduction	30	8.3
transcription	50	13.8
Transport	24	6.6
Unclassified	72	19.9
Others	10	2.8
Total	362	100.0

B

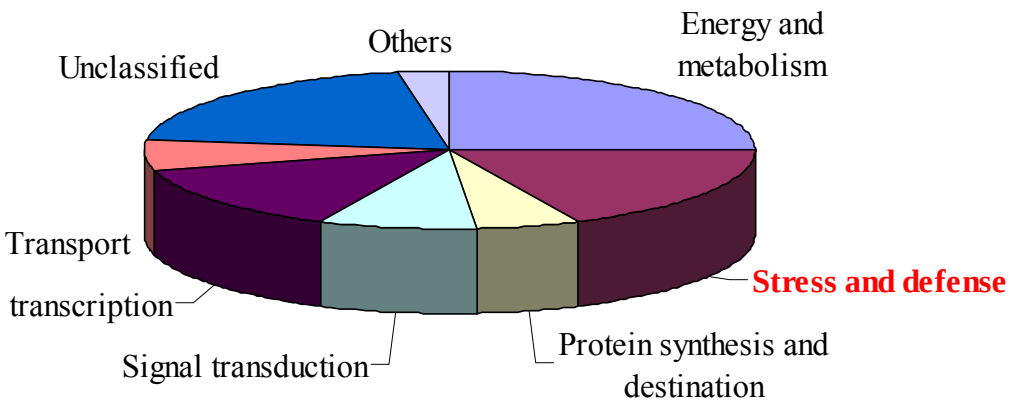


Figure 19. Functional categorization of genes affected in *35S:AtRPD3A* transgenic plants.

Table 6. Genes involved in stress and defense response (*35S:AtRPD3A* microarray data analysis).

Accession	Function	T/C folder	P value
At3g13380	leucine-rich repeat family protein / protein kinase family protein	0.431	0.011
At4g26080	protein phosphatase 2C ABI1 / PP2C ABI1 / abscisic acid-insensitive 1 (ABI1)	1.562	0.047
At4g25340	immunophilin-related / FKBP-type peptidyl-prolyl cis-trans isomerase-related	1.564	0.013
At5g35940	jacalin lectin family protein	3.577	0.039
At3g12580	heat shock protein 70 putative / HSP70 putative	0.33	0.002
At5g15630	phytochelatin synthetase family protein / COBRA cell expansion protein COBL4	1.545	0.036
At2g14580	pathogenesis-related protein putative	2.085	0.012
At5g17780	hydrolase alpha/beta fold family protein	1.896	0.006
At4g13580	disease resistance-responsive family protein	2.069	0.045
At2g32680	disease resistance family protein	0.593	0.025
At2g25980	jacalin lectin family protein	1.932	0.024
At5g37670	15.7 kDa class I-related small heat shock protein-like (HSP15.7-CI)	2.004	0.05
At1g33790	jacalin lectin family protein	2.073	0.002
At2g14610	pathogenesis-related protein 1 (PR-1)	0.133	0.004
At3g57260	glycosyl hydrolase family 17 protein	0.376	0.046
At5g66400	dehydrin (RAB18)	0.287	0.005
At5g36910	thionin (THI2.2)	0.391	0.004
At1g19050	two-component responsive regulator / response regulator 7 (ARR7)	0.48	0.009
At1g72900	disease resistance protein (TIR-NBS class) putative	0.482	0.046
At1g66100	thionin putative	0.496	0.012
At4g37220	stress-responsive protein putative	0.551	0.01
At1g56300	DNAJ heat shock N-terminal domain-containing protein	0.567	0.007
At2g43620	chitinase putative	0.582	0.024
At2g26710	cytochrome P450 putative	0.643	0.012
At5g25610	dehydration-responsive protein (RD22)	0.65	0.022
At4g19530	disease resistance protein (TIR-NBS-LRR class) putative	0.654	0.045
At1g63750	disease resistance protein (TIR-NBS-LRR class) putative	0.655	0.004
At5g06870	polygalacturonase inhibiting protein 2 (PGIP2)	0.656	0.049
At1g66280	glycosyl hydrolase family 1 protein	1.977	0.024
At3g05890	hydrophobic protein (RCI2B) / low temperature and salt responsive protein (LTI6B)	2.186	0.005
At2g17880	DNAJ heat shock protein putative	2.366	0.049
At1g05250	peroxidase putative	2.661	0.003
At1g19940	glycosyl hydrolase family 9 protein	4.925	0.025
At3g01190	peroxidase 27 (PER27) (P27) (PRXR7)	2.519	0
At2g24980	proline-rich extensin-like family protein subcellular localization cell wall	2.735	0.037
At4g26010	peroxidase putative	7.274	0.04
At5g42180	peroxidase 64 (PER64) (P64) (PRXR4)	1.524	0.009
At5g15180	peroxidase putative	2.085	0.046
At5g64100	peroxidase putative	1.536	0.013
At3g26200	cytochrome P450 71B22 putative (CYP71B22)	1.544	0.017
At3g30775	proline oxidase mitochondrial / osmotic stress-responsive proline dehydrogenase (POX) (PRO1) (ERD5)	0.654	0.006
At1g17170	glutathione S-transferase putative	0.3	0.009
At3g45970	expansin family protein (EXPL1). Cell wall	0.305	0.007
At5g56500	chaperonin putative	1.585	0.014
At2g32120	heat shock protein 70 family protein / HSP70 family protein	0.661	0.034
At5g48570	peptidyl-prolyl cis-trans isomerase putative / FK506-binding protein putative	0.492	0.021
At1g01860	dimethyladenosine transferase (PFC1)	1.891	0.045
At1g58370	glycosyl hydrolase family 10 protein / carbohydrate-binding domain-containing protein. Cell wall, subcellular localization	1.607	0.036
At4g13660	pinoresinol-lariciresinol reductase putative	2.19	0.015
At4g02330	pectinesterase family protein	0.492	0.043
At1g70090	glycosyl transferase family 8 protein	0.505	0.017
At4g27830	glycosyl hydrolase family 1 protein	0.515	0.004
At1g02850	glycosyl hydrolase family 1 protein	0.533	0.003
At3g48720	transferase family protein	0.534	0.044
At1g05560	UDP-glucose transferase (UGT75B2)	0.416	0.038
At5g57560	xyloglucan:xyloglucosyl transferase / xyloglucan endotransglycosylase / endo-xyloglucan transferase (TCH4)	0.428	0.022
At2g38080	laccase putative / diphenol oxidase putative	1.544	0.039
At1g49860	glutathione S-transferase putative	1.69	0.009
At1g78340	glutathione S-transferase putative	1.714	0.001
At5g01210	transferase family protein	1.733	0.013
At1g53680	glutathione S-transferase putative	3.015	0.009
At2g39980	transferase family protein	2.151	0.003
At5g66690	UDP-glucoronosyl/UDP-glucosyl transferase family protein	1.524	0.011
At5g62340	invertase/pectin methylesterase inhibitor family protein	2.023	0.015
At5g52640	heat shock protein 81-1 (HSP81-1) / heat shock protein 83 (HSP83)	0.49	0.017
At4g35350	cysteine endopeptidase papain-type (XCP1)	1.597	0.014

DISCUSSION

AtRPD3A and AtRPD3B can be Induced by JA, Ethylene and Wounding

Our first set of experiments was to characterize the spatial expression profile of *AtRPD3A* and *AtRPD3B* and their response to the different plant hormones. Therefore, the expression of *AtRPD3A*:*GUS* and *AtRPD3B*:*GUS* was characterized using GUS histochemical staining as well as GUS quantitative assay. The *AtRPD3A* promoter produced GUS activity in all organs analyzed in the *AtRPD3A*:*GUS* transgenic plants. This result is in accordance with the pattern of the *AtRPD3A* gene transcript accumulation observed by RNA gel blot analysis (Wu et al, 2000b; Plant Chromatin Database, http://chromdb.org) suggesting that *AtRPD3A* is constitutively expressed in *Arabidopsis*. The *AtRPD3B* promoter, however, drove relatively weaker GUS expression in the leaves, stems, flowers and siliques compared to *AtRPD3A* promoter. No GUS expression was detected in the stamens and seeds in *AtRPD3B:GUS* plants and this observation is consistent with the report that *AtRPD3B* transcript is expressed at a low level in *Arabidopsis* (Wu et al, 2000). The cis-element analysis revealed that the *AtRPD3A* and *AtRPD3B* promoters contain *cis*-elements involved in different hormone and stress response pathways, including ABA, ethylene, salicylic acid (SA) and wounding. Furthermore, we have shown that *AtRPD3A*:*GUS* and *AtRPD3B*:*GUS* expression was induced by JA, ethylene and wounding. Our recent study revealed that *AtRPD3A*:*GUS* also can be induced by the pathogen *A. brassicicola* (Zhou et al, 2005). RT-PCR analysis confirmed that the expression of *AtRPD3A* and *AtRPD3B* could be

induced by JA and ethylene. *AtRPD3B*:*GUS* can also be induced by GA. However, *AtRPD3B* promoter does not contain a JA responsive motif, suggesting that a novel JA responsive motif that is not described in the current database might be functional in this promoter.

Previously, study of AtRPD3A indicated that it appeared to be functionally involved in many developmental processes (Wu et al, 2000b; Tian and Chen, 2001; Tian et al, 2003) as well as ethylene, JA, and pathogen response (Zhou et al, 2005). In addition, the expression of *AtRPD3A* could be induced by JA and ethylene implying that *AtRPD3A* may play a role in the plant defense response (Zhou et al, 2005). Our mcroarray data analysis shows that a large portion (18.2%) of genes affected in *35S:AtRPD3A* plants are involved in plant stress and defense response. The finding that *35S:AtRPD3A* plants were more resistant to *A. brassicicola,* whereas *AtRPD3A*-RNAi plants were more sensitive to this pathogen than wild type, further supports the hypothesis that *AtRPD3A* is involved in the plant defense response (Zhou et al, 2005). The induction of plant defense responses by pathogen infection involves the action of the plant hormones ethylene, JA and salicylic acid (SA) (Wang et al, 2002). Our study suggests that AtRPD3A might have a role in ethylene and JA-mediated defense response (Zhou et al, 2005). Recent studies indicated that the transcription factor, ERF1, acts downstream of the junction between ethylene and JA pathway, and it is a key element in the integration of both signals for the regulation of defense response genes (Lorenzo et al, 2003). The study in our lab demonstrated that *AtRPD3A* might act upstream of ERF1 in JA and ethylene signaling in plant defense and ERF1 may not be the direct target of AtRPD3A (Zhou et al, 2005).

The deletion of the *AtRPD3A* promoter from the 5' end revealed that the -*1064RPD3A:GUS* lost the GUS activity, suggesting that the 314 bp region from -1378 bp to -1064 bp is required for promoter activity. This 314 bp region contains ABRE (ABA response) motif, TGACG (JA response) motif, P-box (GA response) motif and WUN (wounding response) motifs. For *AtRPD3B* promoter, the GUS expression was abolished in the -*374RPD3B:GUS* indicating that the 383 bp region between -757 bp to -374 bp is essential for *AtRPD3B* promoter regulation. This 383 bp region contains ERE (ethylene response) motif, P-box (GA response), SA element (SA response) and WUN (wounding response) motif. These motifs might be essential for the promoter activity. Additionally, *AtRPD3B* promoter may harbor a novel JA responsive motif, which was not detected in the promoter sequence analysis. Further mutations could be generated in these regions to define the cis-elements in the promoters.

AtRPD3B Plays Important Role in JA-Mediated Senescence Pathway

We observed that *AtRPD3B* mutants displayed delayed leaf senescence compared with wild type plants. However, delayed leaf senescence was not observed in the AtRPD3A knockout lines (data not shown). Senescence is a developmental event that leads to the death of a cell, an organ, or an organism upon aging. The aging process that results in senescence and limited longevity is a ubiquitous biological phenomenon in most organisms. Plants, especially annual plants, exhibit distinctive aging and senescence processes. However, the systematic study of the genetics of longevity mutations began only recently using *Arabidopsis* as a model system (Grbi and Bleecker, 1995; Oh et al, 1997). The delayed leaf senescence in *AtRPD3B* mutants, *axe1-5*, CS24038 and CS24039,

was demonstrated by measuring changes in typical senescence-associated physiological markers such as chlorophyll content and photochemical efficiency. Our data suggests that AtRPD3B may function normally as a positive regulator of leaf senescence in *Arabidopsis* to regulate the longevity of leaves. Because the *AtRPD3B* mutations affect a wide variety of senescence symptoms, AtRPD3B may function upstream in the regulatory cascade of senescence pathways. HDACs are transcriptional repressors that reduce histone acetylation levels to create condensation of chromatin structure and the repression of gene expression. Deletion of RPD3 in yeast cells results in both upregulating and downregulating gene expression (Bernstein et al, 2000; Kurdistani et al, 2002). It was proposed that deacetylation of histone by RPD3 in certain cases may activate transcription by preventing binding of other repressor complexes in yeast (Bernstein et al., 2000). Our study indicated that decreased expression of *AtRPD3B* in *Arabidopsis* resulted in downregulation some of SAGs. Given the repressive nature of a HDAC, SAG may not be directly regulated by AtRPD3B. It is possible that AtRPD3B induces SAG expression by preventing binding of an unknown transcription repressor that regulates SAG expression directly. Chromatin immunoprecipitation experiments are needed to identify the direct target genes of AtRPD3B.

Although leaf senescence is a developmentally programmed event, the initiation and progression of senescence can be influenced by a range of hormones, such as JA and ethylene. These plant hormones have diverse effects on leaf senescence, affecting parameters such as the onset, progression, and termination of leaf senescence (Woo et al, 2001).

It was observed that *AtRPD3B* was induced by JA application and this upregulation was lost in the *AtRPD3B* mutants, further supporting that AtRPD3B has a role in JA signaling pathway. Furthermore, we observed that the JA response pathway genes, *PDF1.2*, *JIN1*, *ERF1* and *VSP2*, were downregulated in the *AtRPD3B* mutant plants. Therefore, *AtRPD3B* may act as a positive regulator in the JA responsive pathway. We found that *AtRPD3B* mutant lines were hypersensitive to JA. In addition, the *AtRPD3B* mutants were partially infertile and defective of anther dehiscence. Pollen development, anther dehiscence and fertility are key developmental manifestations of JA signaling (Turner and Devoto, 2002). All these data support the idea that the AtRPD3B play an important role in JA signaling pathway. The downregulation of JA responsive genes whereas the hypersensitivity to JA of all the *AtRPD3B* mutants suggested that regulation of JA responsive genes and root response to JA might be independent processes.

The current study supports a role for JA in leaf senescence in *Arabidopsis*. This is based on the demonstration that exogenous application of JA induces leaf senescence, and this induction requires an intact JA signaling pathway. In addition, it has been shown that the endogenous JA level in senescing leaves increased to nearly 500% of that in none-senescent counterpart leaves (He et al, 2002). Thus, AtRPD3B may be involved in leaf senescence via the JA signaling pathway. More recent studies indicated that AtRPD3B could interact with COI1, an F-box protein that was required for JA-mediated plant defense responses (Devoto et al, 2002). F-box proteins are known to interact with SKP1 and cullin proteins to form E3 ubiquitin ligases known as the SCF complexes that selectively recruit regulatory proteins targeted for ubiquitination (Deshaies, 1999).

Coimmunoprecipitation experiments confirmed the interaction in planta of COI1 with SKP1-like proteins and AtRPD3B. Based on the known JA pathway, we propose that in *Arabidopsis*, the SCF^{COI1} complex might modify the activity of a target regulator, AtRPD3B, to regulate expression of JA responsive genes such as *PDF1.2*, *JIN1*, *ERF1* and *VSP2* (Figure 20).

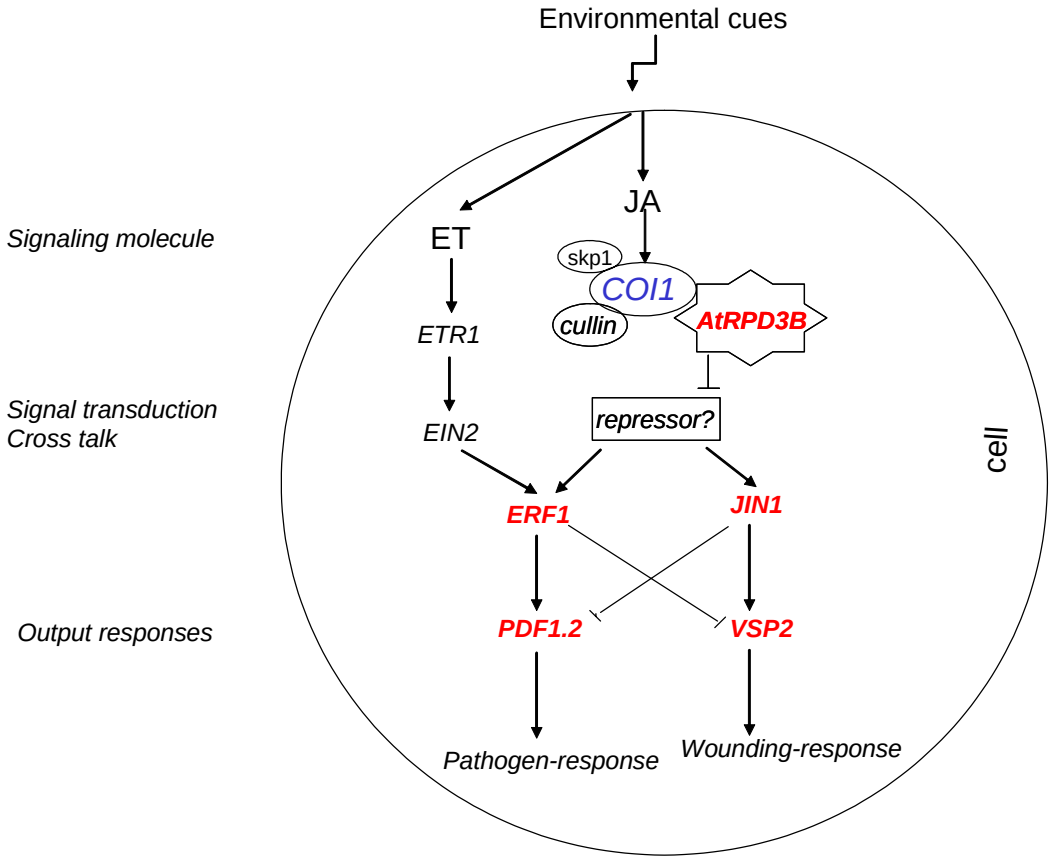


Figure 20. Proposed model of how AtRPD3B and SCF complex regulate JA pathway.

Additionally, genetic studies in *Arabidopsis* indicate that regulated protein degradation is required to control leaf senescence (Oh et al, 1997). It was shown that ORE9, an F-box protein, might limit leaf longevity by removing target proteins that are required to delay the leaf senescence program in *Arabidopsis* via ubiquitin-dependent

proteolysis. It is possible that the SCF^{ORE9} complex interacts with AtRPD3B to achieve the regulation of the key molecules of senescence, such as transcriptional regulators of SAGs or PAGs. Further analysis would be required to reveal whether AtRPD3B can interact with SCF protein ORE9.

AtRPD3B is involved in Autonomous Flowering Pathway

Our observations revealed that the *AtRPD3B* mutants exhibited delayed flowering phenotypes. More recent studies indicated that histone acetylation maybe involved in plant flowering (He et al, 2003; Ausin et al, 2004; Kim et al, 2004). Plant flowering is controlled by environmental conditions and developmental regulation. Molecular genetic studies on the mechanism flowering in *Arabidopsis* have revealed four major pathways: the photoperiod (long-day), autonomous, vernalization, and gibberellin pathways. The autonomous and the vernalization pathways independently regulate the floral transition by repressing FLC expression which is a MADS-box transcription factor that blocks the transition from vegetative to reproductive development (Simpson et al, 1999; Michael et al, 1999; He et al, 2003). All of the *AtRPD3B* mutants flowered much later than their wild type counterparts in long-day and short-day condition, and they are responsive to vernalization treatment, which is typical characteristic of autonomous pathway mutants. The autonomous pathway was identified via a group of mutants that are late flowering under all photoperiods, and are highly responsive to vernalization (Martinez-Zapater and Somerville, 1990; Koornneef et al, 1991). There are six genes in the autonomous pathway: *FLD*, *FCA*, *FPA*, *FY*, *FVE*, and *LUMINIDEPENDENS* (*LD*) (Mouradov et al, 2002; Simpson et al 1999). These genes promote plant flowering by repressing *FLC*. Steady-

state *FLC* mRNA level are much higher in all the *AtRPD3B* the mutants than in the wild types. Increased *FLC* expression is characteristic of other autonomous mutants such as of *fca*, *fpa* and *fve*. The mRNA and protein level of FLC are expressed at much higher levels in *Arabidopsis* after vernalization treatment (Mouradov et al, 2002). We were able to demonstrate that *FLC* is upregulated in the *AtRPD3B* mutant. This observation signifies that *AtRPD3B* regulates flowering by repression of *FLC*. Additionally, *SOC1*, which is the main target gene for converging flowering pathways including the autonomous pathway, vernalization pathway and the long-day pathway, was downregulated in the *AtRPD3B* mutants. *FT*, another target gene downstream to FLC (Pineiro and Coupland, 1998) which functions mainly in photoperiod pathway, was not affected in the *AtRPD3B* mutant, indicating that *AtRPD3B*-mediated promotion of flowering is via specific regulation of *SOC*.

He et al (2003) and Ausin et al (2004) reported that mutations in *FLD* and *FVE* result in hyperacetylation of histones in *FLC* chromatin, up-regulation of *FLC* expression, and extremely delayed flowering. Thus, the autonomous pathway regulates flowering in part by histone deacetylation. However, the hyperacetylation of *FLC* chromatin is only observed in *fld* and *fve* mutants and there is no change in *FLC* acetylation in other mutants of autonomous-pathway genes (*fca*, *fpa*, and *ld*). This supports a model in which FLD and FVE regulate *FLC* expression by a mechanism distinct from other autonomous-pathway genes. Genetic analyses indicate that the autonomous pathway may in fact be composed of genes that control flowering by more than one mechanism (Koornneef et al 1991). Given the centrality of FLC in flowering time control, it is not surprising that FLC is subject to multiple independent regulators. These data indicate that both FLD and FVE

are involved in FLC repression by histone deacetylation, perhaps as components of a HDAC co-repressor complex. In addition, *FLD* encodes a plant homolog of a protein found in histone deacetylase complexes in mammals. *FVE* encodes a predicted protein of 507 amino acids with six WD repeat domains that are frequently found in eukaryotic proteins involved in basic cell regulatory processes (Ausin et al, 2004). There were evidences indicating the increased acetylation level of FLC locus in all the *AtRPD3B* mutants (unpublished data). Summarize all these data together, we propose a model where AtRPD3B, FLC and FVE form a HDAC co-repressor complex to modify the acetylation level of FLC promoting flowering in plants (see Figure 21). Further analysis is required to reveal whether AtRPD3B can interact with FLD and FVE.

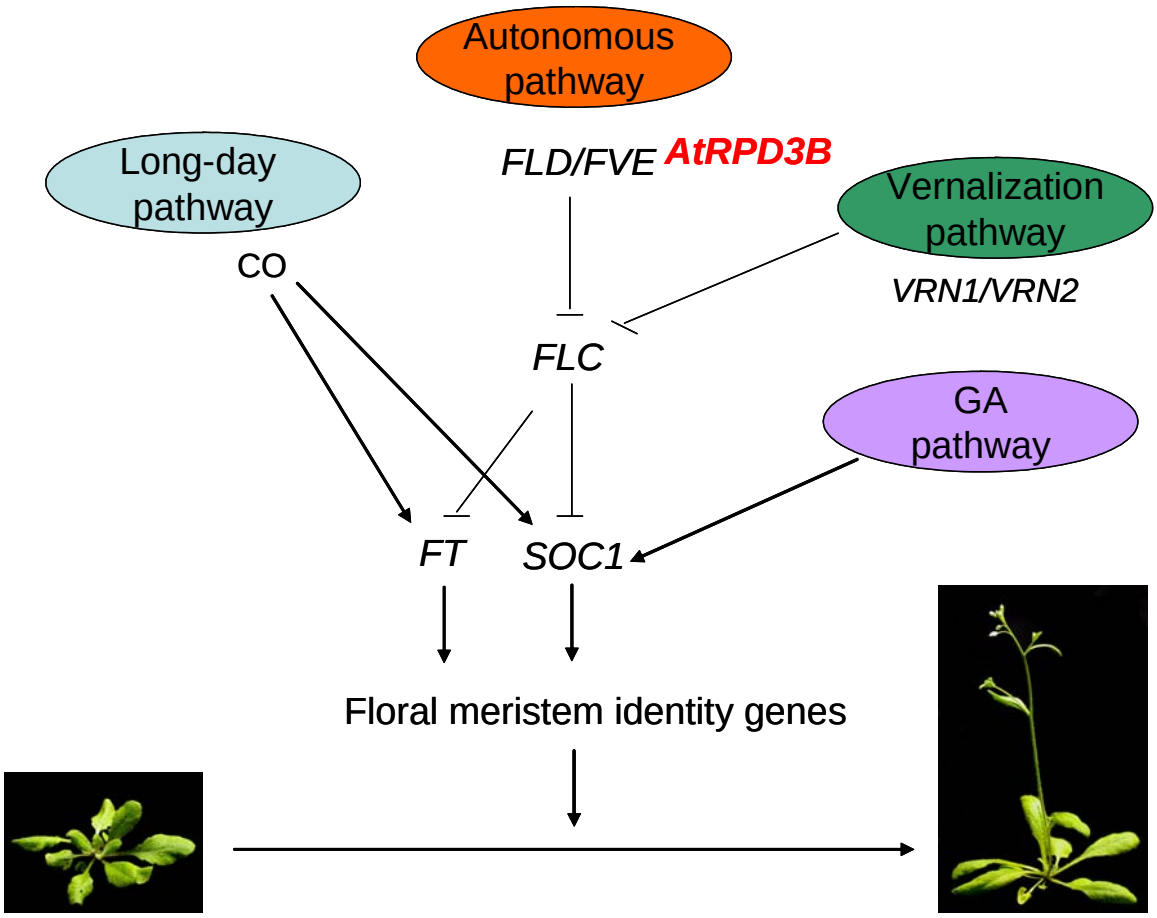


Figure 21. Proposed model of how AtRPD3B is involved in flowering pathway.

Finally, we were able to observe that both AtRPD3A and AtRPD3B were localized into the nuclei of *Arabidopsis* protoplasts. The control of intracellular location has been known as an important regulatory mechanism for HDAC proteins in yeast and mammalian cells (Hirschler-Laszkiewicz et al, 2001). It was demonstrated that mammalian HDA1-type HDACs were mobilized from the cytoplasm to the nucleus by phosphorylation. The localization of AtRPD3A and AtRPD3B to the nucleus supports their roles in transcriptional regulation.

In summary, our study indicates that *AtRPD3A* and *AtRPD3B* can be induced by plant hormones such as JA and ethylene. Their promoters contain several hormone responsive elements. Among the members of HDAC gene families, only *AtRPD3A* and *AtRPD3B* can be induced by JA and ethylene (Zhou et al, 2005). *AtRPD3A* and *AtRPD3B* may regulate gene expression involved in jasmonic acid and ethylene signaling of pathogen response in *Arabidopsis* (Zhou et al, 2005). In addition, AtRPD3B is involved in JA mediated plant senescence pathway and flowering in *Arabidopsis*. AtRPD3A and AtRPD3B belong to the RPD3/HDA1 superfamily and they share 85.4% protein sequence similarty (Wu et al, 2000b) and they may share some common functions as well. On the other hand, they might have specific roles in the regulation of plant developmental pathways.

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